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The intracellular pathway of the acetylcholine-induced contraction in cat detrusor muscle cells

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1 The present study was aimed to investigate intracellular pathways involved in acetylcholine (ACh)-induced contraction in cat detrusor muscle cells

2 Contraction was expressed as per cent shortening of length of individually isolated smooth muscle cells obtained by enzymatic digestion. Dispersed intact and permeabilized cells were prepared for the treatment of drugs and antibody to enzymes, respectively. Using Western blot, we confirmed the presence of related proteins.

3 The maximal contraction to ACh was generated at 10^{-11} M. This response was preferentially antagonized by M_3 muscarinic receptor antagonist ρ-fluoro-hexahydrosiladifenidol (ρF-HSD) but not by the M_1 antagonist pirenzepine and the M_2 muscarinic receptor antagonist methoctramine. We identified G-proteins $G_{q/11}$, G_s, G_0, G_{i1}, G_{i2} and G_{i3} in the bladder detrusor muscle. ACh-induced contraction was selectively inhibited by $G_{q/11}$ antibody but not to other G subunit.

4 The phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor neomycin reduced ACh-induced contraction. However, the inhibitors of the phospholipase D, the phospholipase A_2 and protein kinase C did not attenuate the ACh-induced contraction. ACh-induced contraction was inhibited by antibody to PLC-β_1 but not PLC-β_3 and PLC-γ. Thapsigargin or strontium, which depletes or blocks intracellular calcium release, inhibited ACh-induced contraction. Inositol 1,4,5-triphosphate (IP_3) receptor inhibitor heparin reduced ACh-induced contraction.

5 These results suggest that in cat detrusor muscle contraction induced by ACh is mediated *via* M_3 muscarinic receptor-dependent activation of $G_{q/11}$ and PLC-β_1 and IP_3-dependent Ca^{2+} release.
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Keywords: cat bladder; smooth muscle; detrusor; acetylcholine; muscarinic receptor; G-protein; phospholipase C; calcium

Abbreviations: ACh, acetylcholine; AF-DX 116, (11,11-[[2-(diethyl-amino)methyl]-1-piperidinyl]acetyl)-5,11-di-hydro-H-pyrido[2,3-6][1,4]benzodiazepine-6-)one; BSA, Bovine serum albumin; ρCMB, para-choloromercuribenzoic acid; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; DEDA, dimethyleicosa-dienoic acid; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis (β-aminoehtyl ether)-N,N,N′,N′-tetraacetic acid; ρF-HSD, para-fluoro-hexahydrosila-difenidol; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid; IP_3, inositol 1,4,5-triphosphate; PI, polyphosphoinositide; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SDS, sodium dodecyl sulphate; Tris, 2-aminio-2-hydroxymethyl-1,3-propanediol

Introduction

Acetylcholine (ACh), through its action at muscarinic receptors on smooth muscle cells, is the primary neurotransmitter controlling bladder voiding. Muscarinic receptor density and the sensitivity of bladder smooth muscle to muscarinic stimulation are the greatest in the dome and the lowest in the base, allowing efficient bladder emptying (Ferguson & Christopher, 1996).

Pharmacological, biochemical and genetic data provide ample evidence that muscarinic receptors are heterogeneous in nature. One approach has been to clone of a family of five (m_1–m_5) genes that code for muscarinic receptor subtypes, to transfect clonal cell lines with cDNA coding for individual muscarinic receptor subtypes and then to establish their biochemical properties (Buckley *et al*., 1989). It is well established that the 'odd-numbered' muscarinic receptors (M_1, M_3, and M_5) typically couple *via* the α subunits of the $G_{q/11}$ family, whereas the 'even-numbered' members (M_2, M_4) couple *via* the α subunits of the G_i and G_0 and share the same proposed overall structure and a large degree of protein sequence homology (Bonner *et al*., 1987). This preferential coupling resides at the molecular level mainly in the postulated membrane-proximal regions of the i2 and i3 loops of the different receptors, which are notably different between the 'odd' and 'even' receptor groups, and similar within each of the two groups. The coupling selectivity at the G-protein level is reflected generally, but not exclusively, observed downstream second-messenger pathways activated by the two groups of muscarinic receptors; PLC-β is activated by the 'odd' receptors, that is M_1, M_3 and M_5, whereas adenylyl cylcase is inhibited by the 'even' receptors, that is M_2 and M_4. At least three subtypes of muscarinic receptors (M_1, M_2, and M_3) are pharmacologically distinguishable. The M_1 muscarinic receptor has high affinity for pirenzepine but low affinity for AF-DX 116, whereas the M_2 muscarinic

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receptor has low affinity for pirenzepine but high affinity for AF-DX 116 (Hammer & Giachetti, 1982). A third type of muscarinic receptor is characterized by low affinity for both pirenzepine and AF-DX 116 and by high affinity for 4-DAMP.

In functional studies, stimulation of bladder muscarinic receptors causes smooth muscle contraction. Because bladder appears to contain more than one muscarinic receptor subtype, it is possible that one muscarinic receptor subtype is responsible for bladder detrusor smooth muscle contraction whereas the others are coupled to another parasympathetic associated function, such as influencing neuronal acetylcholine release. It remains to be discovered exactly what factors determine how the specificity of this receptor-effector coupling occurs. It could occur at the level of the coupling of one receptor subtype, through multiple G proteins, to several responses in bladder (Yang *et al*., 2000).

There are evidences on the study of the detrusor muscle that is characterized principally with M_2 and M_3 muscarinic receptors; M_3 muscarinic receptors, coupled to stimulation of phosphoinositide turnover, resulting in the production of IP_3, mediate the direct contractile effects of ACh in the detrusor, wheareas M_2 muscarinic receptors, *via* inhibition of adenylyl cyclase, cause contraction indirectly (Andersson *et al*., 1991; Barras *et al*., 1999; Harriss *et al*., 1995; Hegde *et al*., 1997; Hegde & Eglen, 1999; Sellers *et al*., 2000; Sellers *et al*., 2000; Yamanishi *et al*., 2000). However it was not well studied about intracellular pathways including (a) certain G protein(s) and PLC isozymes in the mediation of muscarinic contraction in detrusor muscle.

In this study, we investigated the characterization of muscarinic receptor subtypes and clarified G protein subtypes or PLC isozymes that were involved in ACh-induced muscle contraction of cat detrusor muscle cells.

Methods

Preparation of bladder detrusor muscle tissue squares

All experimental methods were approved by the Institutional Animal Care and Use Committee of the Chung Ang University in Seoul, Korea. Adult cats of randomized either sex, weighing 2.5–3 kg were used in this study, since ACh-induced maximal contractions of these cells in male ($21.3 \pm 0.2\%$ shortening, $n=45$) were not different when compared to that in female ($21.7 \pm 0.3\%$ shortening, $n=21$). Animals were killed by an overdose of 25% urethane (Aldrich, St. Louis, MO, U.S.A.). After the midline was opened, and the entire urinary bladder was removed, the bladder dome was isolated from the trigone region, the surrounding connective tissue and epithelium were carefully removed from the trigone region. The detrusor smooth muscle was divided into horizontal strips and cut into 0.5 mm thick slices with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA, U.S.A.). The last slices containing serosa were discarded. The slices of smooth muscle layers were placed flat on a wax surface, and tissue squares were made by cutting twice with a 2 mm blade block, the second cut at right angles to the first.

Preparation of dispersed intact and permeabilized smooth muscle cells

Isolated smooth muscle cells were obtained by enzymatic digestion (Sohn *et al*., 1993). The bladder tissue squares were digested in the Krebs solution, containing 10 mM HEPES, 0.09 mg ml^{-1} soybean trypsin inhibitor, 10% BSA, and collagenase 50 unit ml^{-1} and equilibrated with 95% O_2–5% CO_2 to maintain pH 7.45 ± 0.05 at 31°C. The Krebs solution contained (in mM): NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.2, Glucose 11 and $CaCl_2$ 2.5. The solution was gently gassed with 95% O_2–5% CO_2. At the end of digestion period, the tissue was placed over a 350 μm Nitex mesh, rinsed in collagenase-free Krebs solution enough to remove any trace of collagenase, and then incubated in collagenase-free Krebs solution at 31°C and gassed with 95% O_2–5% CO_2. The cells were allowed to dissociate freely in this Krebs solution for 10–20 min. Throughout the entire procedure, care was taken not to agitate the fluid to avoid cell contraction in response to mechanical stress.

Cells were permeabilized, when required, to allow the use of agents such as G protein antibodies or PLC isozyme antibodies or heparin, which do not diffuse across the intact cell membrane (Cao *et al*., 2001; Shim *et al*., 2002; Yang *et al*., 2000). The cells' length after permeabilization was not changed ($75.6 + 0.3$ μm $n=37$ *versus* control $75.8 + 0.4$ μm, $n=46$), and contractile properties was also same. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with an enzyme-free cytosolic buffer of the following composition (in mM): NaCl 20; KCl 100; $MgSO_4$ 5.0; NaH_2PO_4 0.96; EGTA 1.0; and $CaCl_2$ 0.48 and 2% BSA. The cytosolic buffer was equilibrated with 95% O_2–5% CO_2 to maintain a pH of 7.2 at 31°C. Muscle cells dispersed spontaneously in this medium. The cytosolic buffer contained 0.48 mM $CaCl_2$ and 1 mM EGTA, yielding 0.18 μM free Ca^{2+}, as calculated according to Fabiato & Fabiato (1979). After dispersion, the cells were permeabilized by incubation for 5 min in cytosolic buffer that contained saponin (0.1 μg ml^{-1}). After exposure to saponin, the cell suspension was spun at $350 \times g$ and the resulting pellet was washed with saponin-free modified cytosolic buffer that contained antimycin A (10 μM), ATP (1.5 mM) and an ATP-regenerating system that consisted of creatine phosphate (5 mM) and creatine phosphokinase (10 units ml^{-1}). After the cells were washed free of saponin, they were resuspended in modified cytosolic buffer.

Agonist-induced contraction of isolated muscle cells

Medium containing the test agents was added to an aliquot of cell suspension and then the cells were contracted by exposure for 30 s to ACh. When muscarinic antagonists were used, the cells were incubated in appropriate concentrations of the antagonists for 1 min before the addition of ACh. The pretreatment of G proteins antibodies or PLC isozymes antibodies was respectively incubated for 1 h before the addition of ACh in modified cytosolic buffer after permeabilization (Murthy & Makhlouf, 1991; Sohn *et al*., 1993; 1995). After exposure to ACh, the cells suspension was fixed in formalin at a final 0.8% concentration. A drop of the cells

suspension including agonist added in test tube was placed on a glass slide and covered by a cover slip and the edges were sealed with clear nail enamel to prevent evaporation.

The length of 30 consecutive intact cells, encountered at random in each slide, were measured with a phase-contrast microscope (model ULWCD 0.30, Olympus, Tokyo, Japan), and a digital closed-circuit video camera (CCD colour camera, Toshiba, Tokyo, Japan) connected to a Macintosh computer (Apple, Cupertino, CA, U.S.A.) with a software program, Image 1.57 (National Institutes of Heath, Bethesda, MD, U.S.A.). Contraction was expressed as the per cent shortening of individual cells compared with the control cell length.

Determination of muscarinic receptor antagonist potencies

Concentration-response curves were constructed for ACh alone and in the presence of four concentrations of M_1 (pirenzepine), M_2 (methoctramine), or M_3 (pF-HSD) antagonists. The EC_{50} value for ACh alone and in the presence of each antagonist was calculated (Result 1), and Schild plots were then generated to calculate pA_2 and Schild slope values (Figure 1C) according to the method of Arunlakshana & Schild (1959) using a software program (PHARM/PCS Ver. 4) for pharmacological calculations. In all cases slopes did not differ significantly from unity.

Identification of M_2, M_3 muscarinic receptors, PLC isozymes, and G protein subtypes by Western blot

Muscle layers were isolated from bladder detrusor and homogenized in homogenizing buffer containing (mM): Tris HCl 20 (pH 7.5), EGTA 0.5, EDTA 0.5, β-mercaptoethanol 10, leupeptin 10 μg ml^{-1}, and aprotinin 10 μg ml^{-1}. When identification of M_2, M_3 muscarinic receptors, PLC isozyme or G protein is needed, homogenized muscle cells are respectively centrifuged at $14{,}000 \times g$ for 15 min at 4°C (Murthy & Makhlouf, 2000; Sohn *et al*., 1993; 1995; Wang *et al*., 2000; Yang *et al*., 2000). The supernant was subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (M_2 & M_3 muscarinic receptors: 7.5%, PLC isozymes: 5%, G protein subtypes: 12.5% gradient gel). Prestained molecular mass markers were run in an adjacent lane to permit molecular mass determination. The separated proteins were electrophoretically transferred to nitrocellulose (NC) membranes at 80 V in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. Transfer of proteins to the NC membrane was confirmed with Ponseau S staining reagent. The blots were incubated for 1 h at room temperature in phosphate-buffered saline (PBS) containing 5% nonfat dry milk to block non-specific antibody binding. After three or four washes in PBS, the blots were incubated for 1 h at 4°C with isozyme-specific monoclonal antibodies (PLC: 0.5 μg ml^{-1}, G protein: 20 μg ml^{-1}) to PLC isozyme or G

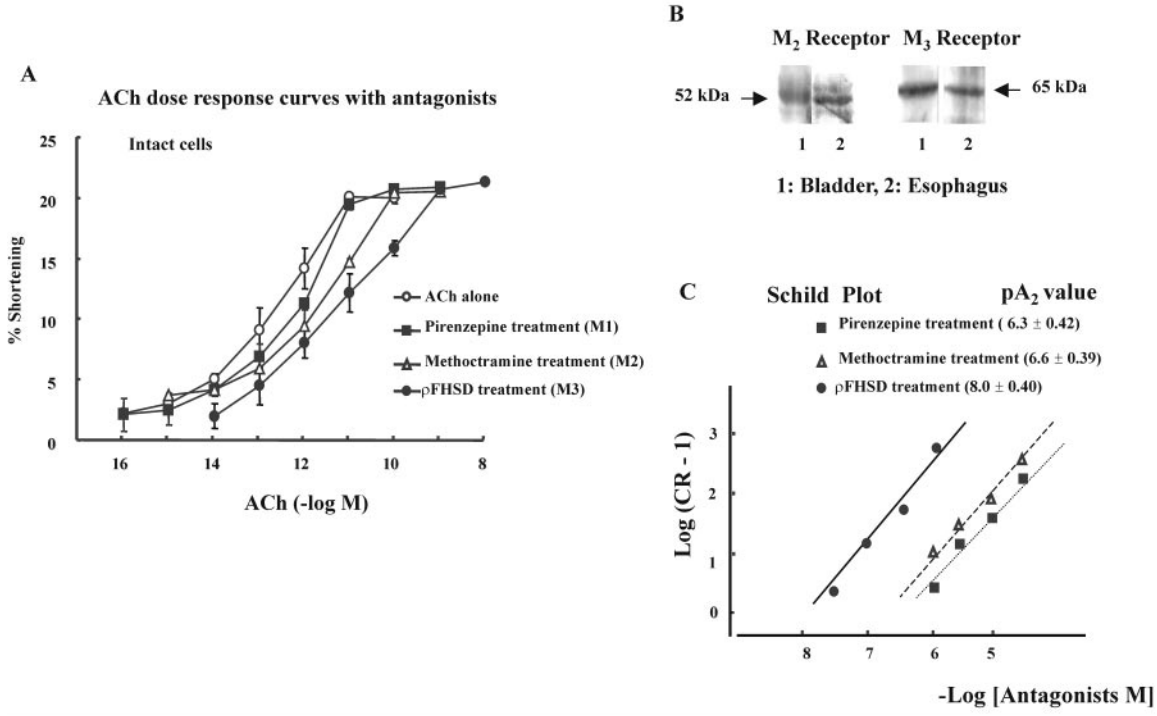

Figure 1 (A) The concentration-response curve of ACh in presence of antagonists (10^{-6} M). Intact bladder detrusor muscle cells were contracted with the increased concentration of ACh alone or after a 1 min pretreatment with 10^{-6} M of the M_1, M_2 or M_3 muscarinic antagonists, pirenzepine, methoctramine or pF-HSD, respectively. The M_3 antagonist pF-HSD produced a significant shift to the right in the ACh concentration-response curve. The values are means ± s.e.mean ($n = 4$). (B) Identification of M_2 and M_3 receptors of cat bladder. The bands of M_2 and M_3 receptors of cat esophagus were presented as control. M_2 and M_3 receptors were all presented in cat bladder smooth muscle. (C) Comparison of antagonistic effects (pA_2) of muscarinic antagonists, pirenzepine (M_1), methoctramine (M_2) and pF-HSD (M_3), in ACh-induced contraction of cat detrusor muscle cells. Each value represents the mean of five experiments. The data points plotted by the method of Arunlakshana & Schild (1959) were fitted by linear regression to obtain the pA_2. All slopes were not significantly different from unity. Each antagonist was used in four different concentrations.

protein with shaking, were removed by washing in PBS containing 0.1% BSA and 0.05% Tween 20, followed by washing three times in PBS containing 0.05% Tween 20 and incubated the blots with horseradish peroxidase-conjugated goat anti-rabbit antibody (1 mg ml^{-1}). Enhanced chemiluminescence reagents detected the G protein bands.

Materials

Methoctramine HCl and ρF-HSD were purchased from Research Biochemical (Natick, MA, U.S.A.); PLC-β antibodies (PLC-β_1, PLC-β_2, PLC-β_3, PLC-β_4, PLC-α_1, α_2, PLC-δ_1, and PLC-δ_2) and G protein antibodies ($G_q - G_{11}$, G_{i1}, G_{i2}, G_{i3}, G_0, and G_s) from Santa Cruz Biotechnology (California, CA, U.S.A.); chelerythrine chloride from LC Laboratories (Woburn, MA, U.S.A.); chemilumiscence agents from NEN™ Life Science (Boston, MA, U.S.A.); PBS from Boehringer Mannheim (Indianapolis, IN, U.S.A.); collagenase from Worthington Biochemicals (Freehold, NJ, U.S.A.); rainbow prestained-molecular weight marker from Amersham (Arlington Heights, IL, U.S.A.); SDS sample buffer and nitrocellulose membrane from BioRad (Richmond, CA, U.S.A.). Horseradish peroxidase-conjugated goat anti-rabbit antibody were obtained from Pierce (Rockford, IL, U.S.A.); Anti-muscarinic M_2 and M_3 receptor serum from Research & Diagnostic Antibodies (Berkeley, CA, U.S.A.); strontium choloride, ρCMB, neomycin, saponin, EGTA, EDTA, HEPES, soybean trypsin inhibitor, BSA, pirenzepine, Ponseau S, leupeptin, aprotinin, and β-mercaptoethanol from Sigma (St. Louis, MO, U.S.A.); thapsigargin and DEDA from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were of the highest purity or molecular biology grade available from commercial sources.

Data analysis

Data are expressed as the means ± standard errors mean (s.e.mean). Statistical differences between groups were determined by Student's *t*-test. Differences between multiple groups were tested using ANOVA for repeated measures and checked for significance using Scheff's *F* test.

Results

Characterization of muscarinic receptors mediating ACh-induced contraction

The mean length of detrusor muscle cell ($n=4$) was 75.6 ± 0.70 μm. ACh produced concentration-dependent contraction of the bladder detrusor muscle cell of cat (Figure 1A). The contraction in response to ACh was not significantly affected by the M_1 antagonist pirenzepine (10^{-6} M) or M_2 antagonist methoctramine (10^{-6} M). In contrast, the M_3 antagonists ρF-HSD (10^{-6} M) caused a significant parallel shift to the right in the concentration-response curve to ACh without alteration of the maximum response. In the presence of ρF-HSD, the negative logarithm value of the EC_{50} of ACh ((11.3 ± 0.26), $n=4$) was significantly greater than that of the control (13.1 ± 0.14), pirenzepine (12.3 ± 0.29), or methoctramine (12.3 ± 0.17), respectively. Esophagus was used as control when investigating the presence of M_2 or M_3 muscarinic receptors. As shown in Figure 1B, M_2 and M_3 muscarinic receptors were presented in cat bladder smooth muscle. (Figure 1B). In addition, it was further shown that, zusing four different concentrations of each antagonists, the pA_2 value of M_3 antagonist was greater than that of M_1 or M_2 antagonist, which suggests that the contraction of detrusor muscle cells may be primarily mediated by the activation of muscarinic M_3 receptors (Figure 1C). The slope was not different from the unity, indicating that each antagonist is competitive.

G protein involved in contractile response to ACh

Identification of G protein subtypes by Western blot Solubilized tissue samples were loaded into the 12.5% SDS polyacrylamide gel electrophoresis system, transferred to NC membrane and incubated with each G protein antibody at a 1 : 1000 concentration for 1 h with continuous shaking. Bands corresponding to $G_{q/11}$ (42 kDa), G_S (46 kDa), G_0 (40 kDa), G_{i1}, G_{i2}, and G_{i3} (40 kDa) protein were identified (Figure 2).

Clarification of G protein subtypes by use of G protein antibodies To identify the specific G proteins involved in bladder detrusor contraction, we used G protein antibodies developed by Spiegel (Goldsmith *et al*., 1987; Shenker *et al*., 1991) and Sternweis (Gutowski *et al*., 1991). These antibodies block receptor-induced activation of G protein by binding to the terminal peptide region of the G protein that interacts with the receptor. The cells were permeabilized with saponin to allow diffusion of the antibody into the cytosolic region of the cell membrane (1 : 200). ACh-induced contraction in permeabilized muscle cells was significantly inhibited by antibody to $G\alpha_{q/111}$ (Figure 3B, $^*P<0.05$), but not by antibodies $G\alpha_{il-3}$, $G\alpha_5$ or G_0 antibody. These data suggest that ACh-induced contraction of detrusor occurs *via* PTX-insensitive $G_{q/11}$ protein.

Involvement of phospholipase in ACh-induced contraction

Effect of phospholipase inhibitors on ACh-induced contraction In search of ACh-activated phospholipase, we examined the effects of phospholipase A_2 inhibitor DEDA (Sohn *et al*.,

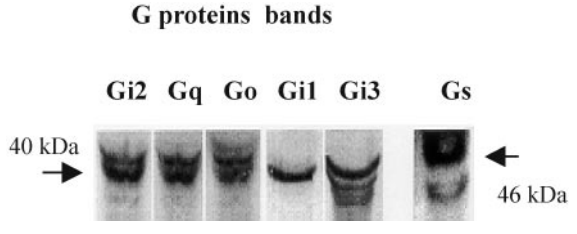

Figure 2 Identification of G-protein in detrusor muscle. Plasma membranes isolated from dispersed bladder muscle cells were homogenized as described in Methods. Membrane proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and then probed with G protein-specific antibodies and anti-rabbit IgG conjugated to horseradish perosicase. Enhanced chemiluminescence reagents identified the G protein bands. Bands corresponding to G_s (46 kDa), G_i group (40 kDa), G_0 (40 kDa) and G_q group (42 kDa) were detected.

1994b), PI-PLC inhibitor neomycin (Delegge *et al*., 1993), and phosphatidylcholine-specific PLD inhibitor rCMB (Sohn *et al*., 1993). ACh-induced contraction of the bladder was reduced by the 10 min preincubation of neomycin (10^{-6}, 10^{-5} or 10^{-4} M, $^{*}P<0.05$, $^{**}P<0.01$ in Figure 4). However, pretreatment for 1 min with different concentrations (10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M) of ρCMB or DEDA did not affect the ACh-induced contraction. These data suggest that detrusor muscle cells contraction is mediated by PI-PLC.

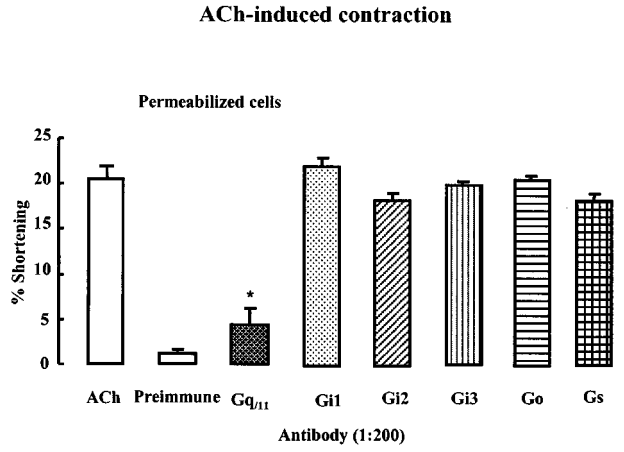

Figure 3 Role of G-protein in ACh-induced contraction. Muscle cells were permeabilized by brief exposure to saponin to allow diffusion of antibodies into the cytosolic side of the cell membrane. Preimmune antiserum did not have any effects on cells. ACh-induced contraction of bladder detrusor muscle cells was inhibited after a 60 min preincubation in cytosolic medium that contained $G_{q/11}$ antibody but not any other G-protein antibodies. Each point is the mean ± s.e.mean with 30 cells counted for each ($n=4$). These data suggest ACh-induced contraction occurs *via* $G_{q/11}$ protein. $^{*}P<0.01$ by ANOVA.

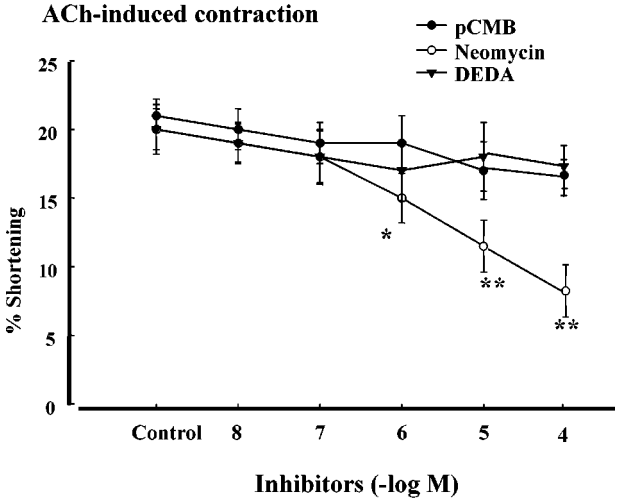

Figure 4 Effects of phospholipases inhibitors on ACh-induced contraction. Intact detrusor muscle cells were contracted with the maximally effective concentration of ACh (10^{-10} M) alone (control) or after 1 min preincubation with PLD inhibitor pCMB ($10^{-8}-10^{-5}$ M) and with PLA_2 inhibitor DEDA ($10^{-8}-10^{-5}$ M). PLC inhibitor neomycin (10^{-6}, 10^{-5} or 10^{-4} M) reduced the ACh-induced contraction. The values are the means ± s.e.mean ($n=5$). $^{*}P<0.05$, $^{**}P<0.01$ by ANOVA.

Identification of PLC isozymes by Western blot Western blot analysis of homogenates obtained separately from dispersed circular muscle using monoclonal antibodies to the main PLC types demonstrated the presence of immunoreactive protein bands corresponding to 150 kDa with PLC-β_1 and PLC-β_3 antibody, and 145 kDa with PLC-γ_1 antibody (Figure 5A).

Clarification of PLC isozymes by use of PLC antibodies To identify the specific PLC isozyme that was involved in ACh-induced detrusor contraction, we used PLC isozyme antibodies in permeabilized cells. When antibodies were used, the permeabilized cells incubated with the antibody at a 1 : 200 dilution for 1 h before addition of ACh (Bitar *et al*., 1992). The results showed that ACh-induced permeabilized cell contraction was significantly reduced by a 1 h preincubation with PLC-β_1 antibody, but not by PLC-β_3 or PLC-γ_1 antibody (Figure 5B, $^{***}P<0.001$).

PKC or IP_3 receptor involvement in ACh-induced contraction

Figure 6A shows that ACh (10^{-10} M)-induced contraction of bladder detrusor muscle cells is not affected by the PKC inhibitor chelerythrine (10^{-5} M), suggesting that ACh-induced detrusor muscle contraction is independent with PKC activation.

Incubation with IP_3 receptor antagonist heparin (10 μg ml^{-1}) for 1 min, inhibited ACh-induced contraction in permeabilized muscle cells (Figure 6B, $^{**}P<0.01$). These data shows that ACh-induced bladder contraction occurs *via* IP_3-dependent Ca^{2+} release from intracellular stores receptor activation but not *via* PKC-dependent mechanisms.

Effect of intracellular Ca^{2+} on contraction of detrusor muscle cells

We examined the dependence of ACh-induced contraction on the presence of extracellular Ca^{2+} or intracellular Caa^{2+}. When Ca^{2+} is replaced by 4 mM Sr^{2+}, which blocks contraction mediated by Ca^{2+} release from intracellular stores, bladder detrusor muscle contraction in response to ACh (10^{-10} M) is significantly reduced (Figure 7A, $^{**}P<0.01$). When intact cells were incubated in Ca^{2+}-free medium containing 2 mM EGTA, ACh-induced contraction was not affected. These data suggest that detrusor muscle contraction in response to ACh depends on intracellular Ca^{2+} release. This hypothesis was further confirmed in Figure 7B, in which cell contraction is significantly reduced by prolonged incubation in thapsigargin (2 μM, 30 min preincubation) which depletes IP_3-sensitive and IP_3-insensitive Ca^{2+} stores ($^{*}P<0.05$).

Discussion

Characterization of muscarinic receptors mediating ACh-induced contraction

ACh acts through muscarinic receptors on the cell membrane, which are known to be linked to GTP binding proteins (Bonner, 1992; Casey & Gilman, 1988; Hosey, 1992). The pharmacological classification is based on different receptor

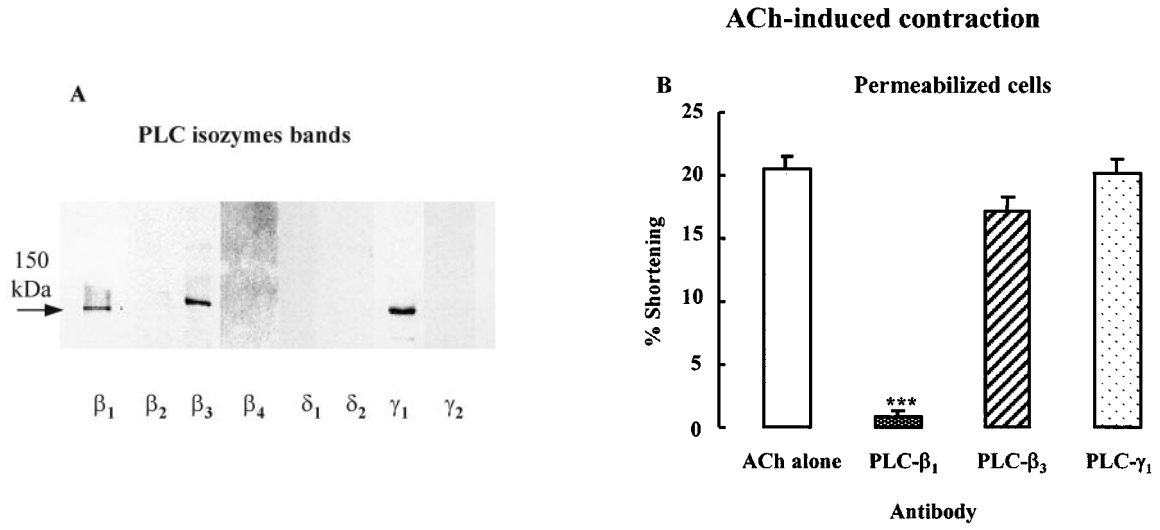

Figure 5 Detection and involvement of PLC isozyme in ACh-induced contraction in detrusor muscle cells. (A) Immunoreactivity of PLC isozymes in homogenates obtained separately from bladder detrusor muscle. After transfer to nitrocellulose membranes, immunoreactive protein bands corresponding to PLC-β_1 and β_3 (150 kDa) and PLC-γ_1 (145 kDa) are identified. (B) The permeabilized bladder muscle cells by brief exposure to saponin were pretreated for 1 h by PLC-β isozymes antibodies and then contracted by ACh (10^{-10} M). These results show that inhibition to ACh-induced contraction by PLC-β_1 antibody was significant (***$P<0.001$ by ANOVA). Values are the means$\pm$s.e.mean of four experiments. Consequently, ACh-induced contraction depends on PLC-β_1 activation.

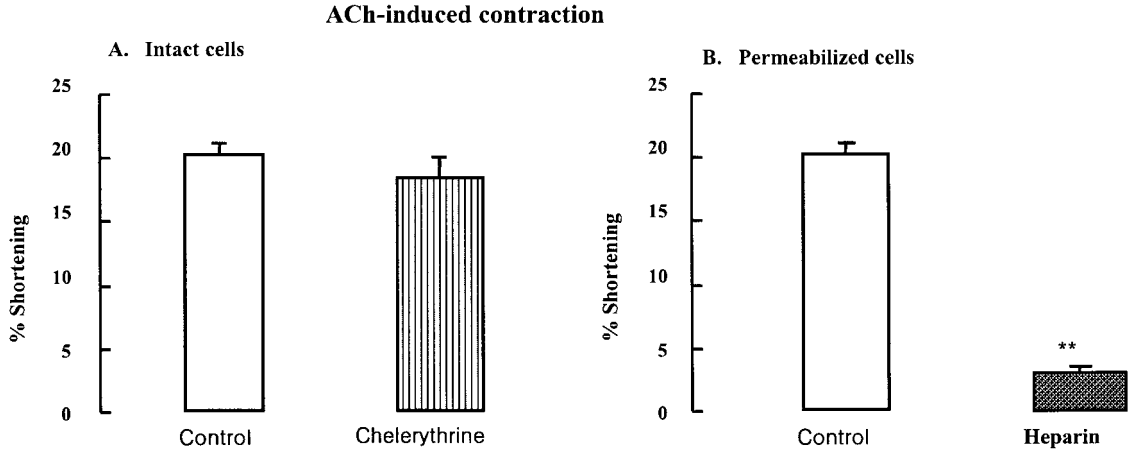

Figure 6 Effect of the preincubation of chelerythrine or heparin on ACh-induced contraction. (A) Contraction of intact bladder muscle cells in response to a maximally effective concentration of ACh (10^{-10} M) alone (control) was not blocked by PKC inhibitor chelerythrine (10^{-5} M) ($n=4$). (B) The permeabilized muscle cells by brief exposure to saponin were contracted with ACh (10^{-10} M) alone (control). IP_3 receptor antagonist heparin (10 mg ml^{-1}, 1 min) significantly reduced the ACh-induced contraction ($n=4$). These data suggest that bladder detrusor contraction in response to ACh depends on IP_3 formation but not PKC activation. The values are the mean$\pm$s.e.mean. **$P<0.01$ by Student's t-test.

affinities for selective antagonists such as pirenzepine ($M_1<M_2=M_3$) (Doods *et al*., 1987; Hammer *et al*., 1980; Hammer & Giachetti, 1982), methoctramine and AF-DX 116 ($M_2<M_1<M_3$) (Melchiorre *et al*., 1987), hexa-hydro-sila-difenidol ($M_1=M_3<M_2$) (Giraldo *et al*., 1988b) and ρF-HSD ($M_3<M_1<M_2$). Subsequently, five distinct ACh muscarinic receptors were cloned. Three (m_1, m_2, m_3) of the five subtypes of muscarinic receptors were recognized through radioligand binding and functional data (Hosey, 1992). A comparison of muscarinic receptors subtypes showed that the antagonist binding properties of the m_1, m_2 and m_3 cloned receptors and their pattern of expression in various tissues corresponded closely to those of the pharmacologically defined M_1, M_2 and M_3 receptors, respectively (Buckley *et al*., 1989; Hosey, 1992). It has been suggested that human esophageal smooth muscle cells express muscarinic receptor subtypes M_1 through M_5 (Wang *et al*., 2000). The M_3 receptor is known as the major muscarinic subtype in the animal bladder responsible for detrusor contraction (Tong & Cheng, 2002). The M_2 receptor is quantitatively the dominant muscarinic subtype in animal

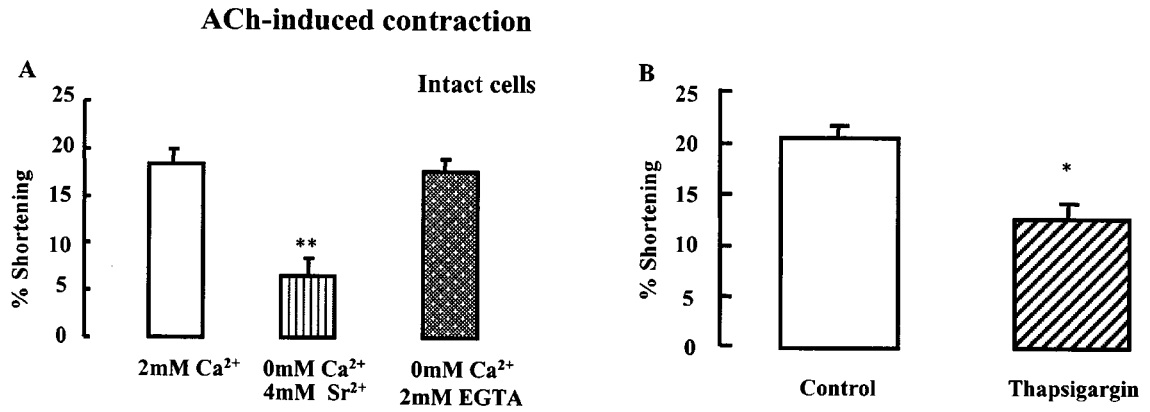

Figure 7 Effect of Ca^{2+} on ACh-induced bladder contraction. (A) The contraction in response to maximally effective ACh (10^{-10} M) was significantly reduced when intact detrusor muscle cells were incubated in Ca^{2+}-free medium containing 4 mM Sr^{2+}. Conversely, Ca^{2+}-free medium containing 2 mM EGTA had no effect ($n=4$, $^{**}P<0.01$ by ANOVA). (B) The cells were contracted with the maximally effective concentration of ACh (10^{-10} M) alone (control) in 2 mM Ca^{2+} medium. ACh-induced contraction of bladder muscle cells was blocked by a 30 min preincubation of thapsigargin (2 mM). ACh-induced contraction mainly uses intracellular Ca^{2+}. The values are the means ± s.e.mean ($n=4$). $^{*}P<0.05$ by Student's *t*-test.

bladders (Tong *et al*., 1999). The presence of the M2 and M3 receptor in bladder or parotid of human, rabbit, guinea-pig, and rat was confirmed (Wang *et al*., 1995; 2000). In this study, we found the presence of M_2 and M_3 receptors in cat bladder smooth muscle.

Methoctramine is the prototype of the polymethylene tetramine class of muscarinic receptor antagonists and is reported to display selectivity toward cardiac M_2 muscarinic receptors (Giraldo *et al*., 1988a; Melchiorre *et al*., 1987). The drug ρF-HSD is a hexahydro-sila-difenidol analogue that has recently been shown to possess considerable selectivity for the smooth muscle M_3 muscarinic receptor in guinea-pig ileum.

In this study, there was no difference of the maximal response between male and female. In bladder strips from females were more sensitive to carbachol than those from males, but there were no differences in sensitivity to electrical field stimulation, as well as no differences in pA_2 values of muscarinic antagonists between bladder strips from male and female rats (Longhurst & Levendusky, 2000). The reason for the different sensitivity may be the use of different species, maximum dose, muscle types (tissue strips and isolated cells). Maximal contraction in isolated cells was produced around 0.1–10 nM range, similar to esophagus, gall bladder and lower esophageal sphincter's maximal contraction, in other isolated cells of other tissues, the potency of the isolated cells was similar about 3–4 log units higher than that of muscle strips at μM ranges (Biancani *et al*., 1994; Sohn *et al*., 1997; 2001; Yu *et al*., 1998). The bladder detrusor muscle cells contraction to ACh in our experiments is significantly reduced by pretreatment of M_3 receptor antagonist, ρF-HSD. However, M_1 receptor antagonist pirenzepine or M_2 receptor antagonist methoctramine did not reduce contraction but rather shifted the ACh concentration-response curve. It was confirmed by pA_2 values; the value of M_3 antagonist was greater than that of M_1 antagonist or M_2 antagonist. It is possible that detrusor muscle contraction is mainly mediated by the activation of muscarinic M_3 receptor. This is consistent with the view that M_3 receptors appear to mediate direct contraction, whereas M_2 receptors act only after selective M_3 inactivation or act indirectly reserving sympathetically (i.e. β-adrenoceptor)-mediated relaxation (Hegde *et al*., 1997; Hegde & Eglen, 1999).

G protein characterization of receptor mediating contractile response to ACh

Western blot analysis of solubilized membrane fractions derived from dispersed bladder detrusor muscle cells demonstrated the presence of a full complement of G proteins: $G_{q/11}$, G_s, G_0, G_{i1}, G_{i2}, and G_{i3}. Additionally, after the muscle cells were permeabilized by brief exposure to saponin, contraction to ACh was significantly reduced by a 1 h preincubation of $G_{q/11}$ protein antibody but not G_5, G_i, or G_0 protein antibody. Therefore, our data suggest that bladder detrusor M_3 receptors may be linked to a G protein of the G_q class. These results were consistent with those that reported the involvement of two members of the G_q class of G α subunits, G_q and $G_{\alpha 11}$, in PTX-resistant coupling to PLC activation. It appears that the contraction of detrusor muscle depends on the activation of M_3 muscarinic receptors, linked to $G_{q/11}$ type of G proteins.

Role of phospholipase C in ACh-induced contraction

The binding of various hormones, neurotransmitters, which include ACh, and peptide growth factors to their cell surface receptors initiates signalling cascades that results in hydrolysis of phosphatidylinositol by phosphoinositide-specific phospholipase C (PI-PLC). As a result of hydrolysis of PLC, IP_3, a Ca^{2+}-mobilizing messenger (Murthy *et al*., 1992; Murthy & Makhlouf, 1991) and diacylglycerol, which activate PKC and results in the phosphorylation of intracellular proteins, are generated (Cockcroft & Thomas, 1992; Nishizuka, 1986; 1992).

Neomycin was reported to inhibit IP_3-dependent Ca^{2+} release in skinned muscle strips of rabbit main pulmonary artery (Kobayashi *et al*., 1989), in gastric circular muscle cells (Delegge *et al*., 1993), and inhibit PI-PLC in esophageal circular cells (Shim *et al*., 2002). Our data suggest that ACh-induced contraction of detrusor muscle results from PI- PLC

activation because ACh-induced contraction of detrusor muscle cells was inhibited by 65% inhibition by neomycin high concentration (10^{-4} M). However, we cannot exclude the possibility that other mechanism may be involved, since the remaining inhibition portion (35%) is still ineffective by this inhibitor. Another PI-PLC inhibitor U73122 remains to be tested in this contraction. Muscarinic M_3-mediated contraction of detrusor muscle is not antagonized by the phosphatidylcholine-specific PLD antagonist ρCMB, which was reported to reduce phosphatidic acid production by directly inhibiting phosphatidylcholine-specific PLD. In addition, PLA_2 was also not involved in ACh-induced contraction of this detrusor mucle, but other PLA_2 inhibtors except DEDA may be needed to be clarified. This results of detrusor muscle was consistent with other findings that muscarinic agonist increase in IPs production by inositol phospholipid hydrolysis *via* PLC by M_3 receptor activation (Barras *et al*., 1999; Mimata *et al*., 1995).

Several subtypes of PLC-β (PLC-β_1, PLC-β_2, PLC-β_3, and PLC-β_4) have now been identified that differ in their mode of activation by G proteins (Boyer *et al*., 1992; Lee *et al*., 1992; Smrcka *et al*., 1991). PLC-β_4 is activated by the a-subunits of all five members of the G_q family of G proteins (G_q, G_{11}, G_{14}, G_{15}, and G_{16}). PLC-β_1 and PLC-β_3 are preferentially activated by the homologous α-subunits of G_q and G_{11} (Lee *et al*., 1992; Smrcka *et al*., 1991), whereas PLC-β_2 is preferentially activated by the α-subunit of G_{16}. PLC-β isoforms are also activated by $\beta\gamma$-dimers, with PLC-β_2 and PLC-β_3 being more responsive than PLC-β_1 (Boyer *et al*., 1992). PLC-β isoforms activated by $\beta\gamma$-dimers derived from interaction of ligands with receptors coupled to G_0 or G_i exhibit PTX sensitivity (Simon *et al*., 1991).

Western blot of PLC isozymes in bladder detrusor muscle showed that PLC-β_1, PLC-β_3 and PLC-γ_1 are present. Additionally, contraction in response to ACh in detrusor muscle cells was significantly abolished by PLC-β_1 antibody but not PLC-β_3 or PLC-γ_1 antibody. These results suggest as new finding, not studied yet, in detrusor muscle cells ACh-induced bladder contraction is mediated by M_3 receptor-dependent activation of $G_{q/11}$ and PLC-β_1.

PKC or IP_3 receptor relationship in ACh-induced contraction

Chelerythrine, a benzophenathridine alkaloid is a potent, selective antagonist of the Ca^{2+}/phospholipid-dependent PKC from the rat brain. Half-maximal inhibition (IC_{50}) of PKC occurs at 0.66 μM. Chelerythrine interacted with the catalytic domain of PKC, was a competitive inhibitor with respect to the phosphate acceptor (histone IIIS, $Ki = 0.7$ μM) and a non-competitive inhibitor with respect to ATP. This effect was further evidenced by the fact that chelerythrine inhibited native PKC and its catalytic fragment identically and did not affect [^{3}H]-phorbol-12, 13-dibutyrate binding to PKC. Chelerythrine is >100 times more potent against PKC than against tyrosine protein kinase, cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase (Herbert *et al*., 1990; Hidaka *et al*., 1981). ACh-induced contraction of bladder smooth muscle cells is not significantly affected by chelerythrine (Herbert *et al*., 1990), suggesting that contraction of bladder muscle may be independent on activation of PKC.

The role of PLC/IP_3 in ACh-induced contraction of the bladder is further supported by the finding that, in permeabilized cells, ACh-induced contraction of bladder was inhibited by the IP_3 receptor antagonist heparin. Heparin acts by blocking the binding of IP_3 to its receptor, thereby preventing the release of IP_3-sensitive intracellular Ca^{2+} (Ghosh *et al*., 1988; Kobayashi *et al*., 1989).

Effect of blockade of intracellular Ca^{2+} release on contraction of detrusor muscle cells

To test the possibility that some Ca^{2+} may be released from intracellular storage sites in response to ACh, we examined the effect of thapsigargin on ACh-induced contraction of intact cells. Thapsigargin enhances the release and/or prevents uptake of IP_3-sensitive or -insensitive Ca^{2+}, resulting in depletion of these stores. After 30 min incubation in thapsigargin, ACh-induced contraction of bladder muscle is significantly reduced, suggesting that in this time complete depletion of bladder Ca^{2+} stores may occur (Bian *et al*., 1991; Sohn *et al*., 1994a). The theory that ACh-induced contraction of bladder muscle is dependent on intracellular Ca^{2+} release, is also supported by the finding that the contraction is affected by substituting Ca^{2+} with Sr^{2+}. ACh-Induced contraction of bladder muscle depends on release of Ca^{2+} from intracellular Ca^{2+} stores since it is significantly reduced by incubation in 4 mM Sr^{2+} medium instead of Ca^{2+} but not in Ca^{2+}-free medium containing 2 mM EGTA. Blockade of intracellular Ca^{2+} release was obtained by manipulating intracellular Ca^{2+} stores with strontium. Strontium is thought to displace and replace Ca^{2+} at storage sites in smooth muscle, but is not readily released from these sites by stimulatory agents. Sr^{2+} mimics the influx of extracellular Ca^{2+}, supports a K^+-induced response, which depends on extracellular Ca^{2+} and does not support equally well the response to stimulatory agents (e.g., norepinephrine), which are resistant to blockade of extracellular Ca^{2+} influx (Yasuda & Sakai, 1984). Thus Sr^{2+} may substitute for extracellular Ca^{2+} and support muscle contractions that utilize influx of extracellular Ca^{2+}, while inhibit contractions that depend on release of intracellular Ca^{2+}. These findings are consistent with the hypothesis that detrusor muscle utilizes intracellular Ca^{2+}. Thus Sr^{2+} blocks actions mediated by Ca^{2+} release while it maintains effects mediated by extracellular Ca^{2+} influx (Biancani *et al*., 1987; Hillemeier *et al*., 1991). This result may support the finding that agonists, including carbachol, histamine and ATP, also activated repetitive increases of Ca^{2+} released from Ca^{2+} store (Chambers *et al*., 1996; Masters *et al*., 1999). These data support the view that contraction of detrusor muscle is dependent on PI- PLC and IP_3 and requires the release of intracellular Ca^{2+}.

In conclusion, we have found in this study that in detrusor muscle cell signalling, detrusor muscle contraction to ACh in cat is mediated *via* muscarinic M_3 receptors – $G_{q/11}$ protein coupling and involves the activation of PLC-β_1 and intracellular Ca^{2+} mobilization *via* IP_3 receptor on Ca^{2+} stores.

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