Muscarinic regulation of cytosolic free calcium in canine tracheal smooth muscle cells: Ca^{2+} requirement for phospholipase C activation

¹Chuen Mao Yang, Sheng-Ping Chou, Yen-Yi Wang, Jen-Tsung Hsieh & Richard Ong

Cellular and Molecular Pharmacology Laboratory Department of Pharmacology, Chang Gung Medical College, Kwei-San, Tao-Yuan, Taiwan

1 The relationship between muscarinic receptor-mediated phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown and the increase of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) has been examined in canine cultured tracheal smooth muscle cells (TSMCs).

2 Addition of acetylcholine (ACh) and carbachol led to a 2–3 fold increase in $[\text{Ca}^{2+}]_i$ over the resting level as determined by fura-2, with half-maximal stimulation (EC_{50}) obtained at concentrations of 97 and 340 nM, respectively. Addition of the partial agonist, bethanechol, showed a smaller increase in PIP_2 turnover and $[\text{Ca}^{2+}]_i$ than did ACh or carbachol.

3 Addition of ACh or carbachol to TSMCs that had been prelabelled with [^3H]-inositol led to the rapid (5–15 s) release of inositol mono, bis and trisphosphates IP_1 , IP_2 and IP_3 . The time course of IP_3 accumulation is correlated with the time course of the peak rise in $[\text{Ca}^{2+}]_i$.

4 Inclusion of EGTA lowered the resting $[\text{Ca}^{2+}]_i$ and markedly reduced the extent of the agonist-induced rise in $[\text{Ca}^{2+}]_i$. When assayed under conditions similar to those used for the $[\text{Ca}^{2+}]_i$ measurements, EGTA reduced the muscarinic agonist-stimulated inositol phosphates (IPs) accumulation. Conversely, ionomycin could stimulate IPs accumulation and elevate $[\text{Ca}^{2+}]_i$. The addition of Ca^{2+} (2.7–617 nM) to digitonin-permeabilized TSMCs directly stimulated IPs accumulation.

5 Both Ca^{2+} and guanosine-5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$) stimulated the formation of IPs in digitonin-permeabilized TSMCs prelabelled with [^3H]-inositol. A further calcium-dependent increase in IPs accumulation was obtained by inclusion of either $\text{GTP}\gamma\text{S}$ or carbachol. The combined presence of carbachol and $\text{GTP}\gamma\text{S}$ elicited a synergistic effect on IPs accumulation, with half-maximal stimulation observed at approximately 8 nM free Ca^{2+} .

6 These results indicate that (i) the magnitude of the initial rise in $[\text{Ca}^{2+}]_i$ is directly related to the production of IPs and (ii) the phospholipase C-mediated PIP_2 breakdown in TSMCs is sensitive to regulation by physiologically relevant concentrations of free Ca^{2+} ($[\text{Ca}^{2+}]_i$).

Keywords: Inositol phosphates; tracheal smooth muscle cells; calcium signal; muscarinic agonists; muscarinic receptors

Introduction

The contractile response of tracheal smooth muscle to cholinergic agonists is mediated through muscarinic receptors (Barnes, 1992). Stimulation of muscarinic receptors results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) through activation of phospholipase C (PLC), thus generating two intracellular messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Yang *et al.*, 1991a,b; Coburn & Baron, 1990; Baron *et al.*, 1984; Grandordy *et al.*, 1986; van Amsterdam *et al.*, 1989; Rosenberg *et al.*, 1991). IP_3 causes an increase of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (Hashimoto *et al.*, 1985), and DAG activates protein kinase C (Coburn & Baron, 1990). Since the agonist-induced activation of PLC has been shown in certain tissues to be dependent on guanine nucleotides, a guanine nucleotide binding protein (G protein) may be involved in the transduction of extracellular signals from the receptor binding sites to PLC (Litosch & Fain, 1985; Fain, 1990). A stable analogue of guanosine triphosphate (GTP), guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$), has been previously shown to increase IPs accumulation in permeabilized tracheal smooth muscles of rabbits (Rosenberg *et al.*, 1991) and cows (van Amsterdam *et al.*, 1989). Furthermore, a G protein α subunit purified from bovine brain has been shown to stimulate selectively PIP_2 hydrolysis by a partially purified PLC (Smrcka *et al.*, 1991). However, potentiation of $\text{GTP}\gamma\text{S}$ of agonist-induced IPs accumulation at various free Ca^{2+} concentrations has not

been demonstrated in canine tracheal smooth muscle cells (TSMCs).

In some tissues, an increase in $[\text{Ca}^{2+}]_i$ may regulate intracellular metabolism involving PLC activation (Best, 1986; Eberhard & Holz, 1991). We previously reported that muscarinic receptor activation by carbachol caused an increase in $[\text{Ca}^{2+}]_i$ in canine TSMCs (Yang, 1992). This increase could be due to the release of Ca^{2+} from internal stores or to the entry of Ca^{2+} from outside the cells (Yang, 1992). The presence of extracellular Ca^{2+} was found to be required for the generation of an initial increase, followed by a plateau in $[\text{Ca}^{2+}]_i$ with carbachol-stimulation of TSMCs. Furthermore, in the absence of extracellular Ca^{2+} , carbachol only caused an immediate and transient increase in $[\text{Ca}^{2+}]_i$, the magnitude of which was less than that observed in Ca^{2+} -containing buffer (Yang, 1992). No sustained elevation of $[\text{Ca}^{2+}]_i$ was observed. In permeabilized chromaffin cells (Eberhard & Holz, 1987) and human SK-N-SH neuroblastoma cells (Fisher *et al.*, 1989), the formation of IPs appeared to be induced by micromolar Ca^{2+} as well as by activators of G proteins (Fisher *et al.*, 1989; Eberhard & Holz, 1991). Therefore, the question arises as to whether an influx of external Ca^{2+} links the muscarinic receptors to the cellular responses mentioned above.

In the present study, we demonstrate that exposure of TSMCs to muscarinic agonists could produce a pronounced increase in $[\text{Ca}^{2+}]_i$, that is closely related to the magnitude of IPs accumulation elicited by respective agonists. Both Ca^{2+} signalling and IPs accumulation in intact cells are markedly

¹ Author for correspondence.

influenced by inclusion of the Ca^{2+} chelator, EGTA. In digitonin-permeabilized cells, the increase of PLC activity due to addition of either a muscarinic agonist or GTP γ S, seemed to be regulated by nanomolar concentrations of free Ca^{2+} . Therefore, in TSMCS, the enzymatic machinery underlying generation of the $[\text{Ca}^{2+}]_i$ signal may be sensitive to changes in $[\text{Ca}^{2+}]_i$ within the physiological range.

Methods

Animals

Mongrel dogs, 10–20 kg, purchased from a local supplier, were used throughout this study. Dogs were housed indoors in the animal facilities with automatically controlled temperature and light cycle and were fed standard laboratory chow and tap water *ad libitum*. Dogs of either sex were anaesthetized with pentobarbitone (30 mg kg $^{-1}$, intravenously) and the lungs were ventilated mechanically via an orotracheal tube. The tracheae were surgically removed.

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to methods described previously (Yang, 1990; Yang *et al.*, 1991a,b). The trachea was cut longitudinally through the cartilage rings and the smooth muscle was dissected. The muscle was minced and transferred to dissociation medium containing 0.2% collagenase I, 0.01% DNase I, 0.01% elastase IV, and antibiotics in a physiological solution. The physiological solution contained (mM): NaCl 137, KCl 5, CaCl_2 1.1, NaHCO_3 20, NaH_2PO_4 1, glucose 11 and HEPES 25 (pH 7.4). The tissue pieces were gently agitated at 37°C in a rotary shaker for 1 h. The released cells were collected and the residue was again digested with fresh enzyme solution for an additional hour at 37°C. The released cells were washed twice with DMEM/F-12 medium. The cells, suspended in DMEM/F-12 containing 10% foetal bovine serum (FBS), were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts. The cell number was counted and the suspension diluted with DMEM/F-12 to a concentration of 2×10^5 cells ml $^{-1}$. The cell suspension (2 ml/well) was plated onto 12-well culture plates or 6-well culture plates containing glass coverslips coated with collagen. Culture medium was changed after 24 h and then every 3 days. After 5-days in culture, cells were moved to DMEM/F-12 containing 1% FBS for 24 h at 37°C. Then, the cells were cultured in DMEM/F-12 containing 1% FBS supplemented with IGF-I (10 ng ml $^{-1}$) and insulin (1 μ g ml $^{-1}$) for 12–14 days and used for measurements 3 to 5 days later.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by indirect immunofluorescence using a monoclonal antibody to light chain myosin (Gown *et al.*, 1985). Over 95% of the cells were smooth muscle cells.

Accumulation of inositol phosphates

Effects of muscarinic agonists on the hydrolysis of PIP_2 were assayed by monitoring the accumulation of ^3H -labelled IPs as described by Berridge *et al.* (1983). Cultured TSMCs were incubated with 5 $\mu\text{Ci ml}^{-1}$ of *myo*-[2- ^3H]-inositol at 37°C for 2 days. TSMCs were washed two times with, and incubated in Krebs-Henseleit buffer (KHS, pH 7.4) containing (in mM) NaCl 117, KCl 4.7, MgSO_4 1.1, KH_2PO_4 1.2, NaHCO_3 20, CaCl_2 2.4, glucose 1, HEPES 20 and LiCl 10 at 37°C for 30 min. After the addition of a muscarinic agonist incubation was continued for another 60 min. When antagonists were used, they were applied 10 min before the addition of the agonist. Reactions were terminated by addition of 5% per-

Figure 1 Dependence of the rise in $[\text{Ca}^{2+}]_i$ on acetylcholine (a), carbachol (b), and bethanechol (c) concentrations. Confluent cultures of canine TSMCs on glass coverslips were loaded with 5 μM fura-2/AM and fluorescent measurement of $[\text{Ca}^{2+}]_i$ was carried out in a dual excitation wavelength spectrofluorometer, with excitation at 340 and 380 nm. The dose-dependent curves of the muscarinic agonists ACh, carbachol, and bethanechol-induced initial and sustained $[\text{Ca}^{2+}]_i$ were calculated based on six separated experiments. The results are expressed as mean $\pm$ s.e.mean as the increase above the basal levels. Open symbols represent the initial peaks and closed symbols are the plateau phases.

chloric acid followed by sonication and centrifugation at 3000 g for 15 min.

The perchloric acid soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied to a column of AG1-X8, formate form, 100–200 mesh (Bio-Rad). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate–5 mM sodium tetraborate to eliminate free *myo*-[^3H]-inositol and glycerophosphoinositol, respectively. The fraction of total IPs was eluted with 5 ml of 1 M ammonium formate–0.1 M formic acid. The amount of [^3H]-IPs was determined in a radiospectrometer (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Permeabilized cells

After a prelabelling period, TSMCs were washed two times with PBS and then permeabilized in KGEH buffer containing (mM): K $^+$ glutamate 139, ATP 2, MgCl_2 4, LiCl 10, EGTA 2, HEPES 20 (pH 7.4) and digitonin 10 μM , as described by Eberhard & Holz (1987). Cells were incubated for 5 min at 37°C, then washed two times with KGEH buffer without digitonin. Cells were then incubated in KGEH with 2 mM EGTA for 15 min at 37°C, before CaCl_2 was added to obtain the required free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_f$). The actual

$[Ca^{2+}]_i$ was measured by adding 10 μ M fura-2 (K^+ -salt). Carbachol or ACh (100 μ M) was added and incubated for 60 min at 37°C. Reactions were terminated by adding 5% perchloric acid. The procedures for extraction, separation, and quantification of IPs were the same as those used for intact cells.

Measurement of intracellular Ca^{2+} level

$[Ca^{2+}]_i$ was measured in monolayers with the calcium-sensitive dye fura-2/AM as described by Grynkiewicz *et al.* (1985). Upon confluence, the cells were cultured in DMEM/F-12 with 1% FBS 1 day before measurements were taken. The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 μ M fura-2/AM and were incubated at 37°C for 45 min. At the end of the loading period, the cells were washed twice with the physiological buffer solution (PBS) containing (mM): NaCl 125, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10 and glucose 10, pH 7.4, and then incubated in PBS for a further 30 min to complete dye de-esterification. The coverslip was inserted into a quartz cuvette at an angle of approximately 45° to the excitation beam and placed in the thermostatted holder of a SLM 8000C spectrofluorometer (SLM Aminco, Urbana, IL, U.S.A.). Continuous stirring was achieved with a magnetic stirrer.

Table 1 The maximum increase in inositol phosphates (IPs) accumulation induced by the various muscarinic agonists

Agonist	IPs accumulation (d.p.m. $\times 10^{-4}$)
Control	1.03 $\pm$ 0.05
Acetylcholine	11.48 $\pm$ 0.17
Carbachol	10.75 $\pm$ 0.96
Oxotremorine	4.00 $\pm$ 0.61*
Bethanechol	4.79 $\pm$ 0.35*
Arecoline	4.26 $\pm$ 0.26*

$[^3H]$ -inositol labelled TSMCs, were incubated for 60 min with either acetylcholine (100 μ M), carbachol (100 μ M), oxotremorine (1 mM), bethanechol (1 mM) or arecoline (1 mM). IPs were separated and quantitated. Results are expressed as mean $\pm$ s.e.mean from three separate experiments.

* $P < 0.001$ as compared with cells stimulated by acetylcholine.

Figure 2 Concentration-dependence of muscarinic agonist-evoked inositol phosphates (IPs) accumulation in TSMCs. Muscarinic agonists, acetylcholine ACh (○), carbachol (●) or bethanechol (□) were added to $[^3H]$ -inositol labelled TSMCs and incubated for 60 min. The reactions were terminated by addition of 5% PCA, and IPs were separated and quantitated. Results are expressed as the percentage of maximal response to ACh (116500 $\pm$ 900 d.p.m./well) from at least three separate experiments.

Fluorescence of Ca^{2+} -bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) of the fluorescence at the two wavelengths were computed and used to calculate changes in $[Ca^{2+}]_i$. The ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were determined by lysing the cells with ionomycin (10 μ M) in the presence of PBS containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in a Ca^{2+} -free PBS, respectively. Values obtained were 14.09, 0.96, and 22.07 for R_{max} , R_{min} , and β , respectively. The K_d of fura-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz *et al.*, 1985).

Analysis of data

Data were expressed as the mean $\pm$ s.e.mean of at least four experiments with statistical comparisons based on Student's two-tailed *t* test at a $P < 0.05$ level of significance.

Chemicals

DMEM/F-12 medium and FBS were purchased from J.R. Scientific (Woodland, CA, U.S.A.). Insulin and IGF-I were obtained from Boehringer Mannheim (GmbH, Germany). Fura-2/AM was from Molecular Probes Inc (Eugene, U.S.A.). $Myo-[^3H]$ -inositol (18 Ci mmol $^{-1}$) was obtained from Amersham (Buckinghamshire, U.K.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.).

Results

Relationship between muscarinic agonist concentration and rise in $[Ca^{2+}]_i$

The muscarinic agonists, acetylcholine (ACh), carbachol, and bethanechol, were found to cause a dose-dependent elevation of $[Ca^{2+}]_i$ levels, as measured directly in cultured TSMCs loaded with fura-2 (Figure 1). The resting free $[Ca^{2+}]_i$ in fura-2 loaded TSMCs was 190 $\pm$ 24 nM ($n = 39$). In the presence of extracellular Ca^{2+} (1.8 mM), agonist addition to the fura-2-loaded cells resulted in a rapid increase in $[Ca^{2+}]_i$ and was followed by a decline to a steady value which was significantly higher than the resting $[Ca^{2+}]_i$. The biphasic responses became less pronounced as the agonist concentration was reduced. ACh (10 μ M), carbachol (100 μ M) and bethanechol (1 mM) caused a maximum increase in the $[Ca^{2+}]_i$, reaching a peak of 457 $\pm$ 41, 406 $\pm$ 33, and 363 $\pm$ 35 nM, respectively, ($n = 8$, Figure 1). The initial rise in $[Ca^{2+}]_i$ was very rapid, reaching the peak within approximately 5 s, was transient and declined to a plateau of 350 $\pm$ 33, 339 $\pm$ 25, and 296 $\pm$ 29 nM ($n = 8$) for ACh, carbachol, and bethanechol, respectively, in approximately 30 s. The half-maximal effects (EC_{50}) of ACh, carbachol, and bethanechol to induce the initial peak in $[Ca^{2+}]_i$ were 97 $\pm$ 5 nM, 340 $\pm$ 53 nM, and 53.5 $\pm$ 7.8 μ M, compared with values of 264 $\pm$ 48 nM, 330 $\pm$ 65 nM, and 57.6 $\pm$ 9.0 μ M for the plateau phases, respectively. The plateau persisted until the agonist was displaced by the addition of atropine, when it declined to a level substantially higher than that of the resting levels (data not shown).

Comparison of changes in IPs accumulation and $[Ca^{2+}]_i$ induced by muscarinic agonists

The increase of IPs accumulation appeared to be induced to different extents by the muscarinic agonists ACh, carbachol, oxotremorine, bethanechol, and arecoline in TSMCs as shown in Table 1. Bethanechol, oxotremorine and arecoline

Figure 3 Full and partial muscarinic agonists mobilize the same metabolic pool of Ca^{2+} . (a), To fura-2-loaded TSMCs were added 1 mM bethanechol (Beth), followed by 100 μM acetylcholine (ACh). Note that the addition of ACh after an initial exposure of the cells to bethanechol elicited a further rise in $[\text{Ca}^{2+}]_i$. The initial peak $[\text{Ca}^{2+}]_i$ values obtained with bethanechol and ACh were 464 and 474 nM, respectively. The corresponding plateau $[\text{Ca}^{2+}]_i$ values were 358 and 427 nM. (b) When the cells were first challenged with ACh, the subsequent addition of bethanechol had no effect on $[\text{Ca}^{2+}]_i$. (c and d), Parallel experiments were performed using carbachol (CCh) under the same conditions as those of (a) and (b). (c) The addition of carbachol (100 μM) after an initial exposure to bethanechol (1 mM) elicited a further increase in $[\text{Ca}^{2+}]_i$. The initial peak $[\text{Ca}^{2+}]_i$ values obtained with bethanechol and carbachol were 389 and 419 nM, respectively. The respective plateau $[\text{Ca}^{2+}]_i$ values were 292 and 387 nM. (d) When TSMCs were first exposed to carbachol, the subsequent addition of bethanechol had no effect on $[\text{Ca}^{2+}]_i$. The traces are from one of at least five experiments that gave similar results. Atr, 10 μM atropine.

at maximally effective concentrations induced less increase in IPs than maximal concentrations of ACh and carbachol ($P < 0.001$). The increase of IPs accumulation induced by various concentrations of ACh, carbachol and bethanechol in TSMCs was dose-dependent as shown in Figure 2. Among these agonists, bethanechol at a maximally effective concentration caused the least increase in IPs accumulation. The order of potency for muscarinic agonists with respect to EC_{50} values in TSMCs was: ACh (1.8 μM) > carbachol (7.6 μM) > bethanechol (47 μM). Addition of agonists such as ACh and carbachol, known to stimulate maximally PIP_2 hydrolysis, resulted in an increase of $[\text{Ca}^{2+}]_i$ (Table 2). In contrast, partial agonists of PIP_2 hydrolysis such as bethanechol, oxotremorine and arecoline, were less effective ($P < 0.01$, as compared with that of $[\text{Ca}^{2+}]_i$ stimulated by ACh; Table 2). Once an increase of $[\text{Ca}^{2+}]_i$ had been elicited by addition of either a full or partial agonist, the subsequent addition of the same agonist did not result in a further rise in $[\text{Ca}^{2+}]_i$ (data not shown). However, if TSMCs were first exposed to bethanechol, a partial agonist, the subsequent addition of either ACh or carbachol did result in a further rise of $[\text{Ca}^{2+}]_i$ (Figure 3a and c). If ACh or carbachol was applied first, the subsequent addition of bethanechol failed to increase $[\text{Ca}^{2+}]_i$ further (Figure 3b and d). These results indicated that both full and partial muscarinic agonists mobilize the same cellular Ca^{2+} pools but to different extents.

Effect of EGTA on calcium signalling

Reduction of extracellular Ca^{2+} to approximately 60 nM $[\text{Ca}^{2+}]_i$ by addition of 4 mM EGTA resulted in three distinct consequences (Figure 4). First, the resting $[\text{Ca}^{2+}]_i$ was reduced from 190 ± 24 to 140 ± 20 nM ($n = 20$) within 5 min for fura-2-loaded TSMCs, indicating that extracellular EGTA may deplete the cytoplasmic Ca^{2+} , as previously noted for aortic smooth muscle cells (Capponi *et al.*, 1985). Second, the magnitude of the initial rise of $[\text{Ca}^{2+}]_i$ by addition of either ACh or carbachol was reduced to 200 ± 14 and 203 ± 24 nM ($n = 10$, Figure 4b and e) from normal values of approximately 457 ± 41 and 396 ± 33 nM (Figure 4a and d), respectively. As a third consequence, the plateau phase of $[\text{Ca}^{2+}]_i$ was abolished and the peak declined to the resting levels within 50 s after the application of the agonist (Figure 4b and e). Subsequent addition of ionomycin (50 nM) to these cells did evoke a further increase of $[\text{Ca}^{2+}]_i$, presumably due to the discharge of other intracellular stores of Ca^{2+} (Figure 4b and e). Upon the addition of Ca^{2+} (4 mM) to EGTA-treated TSMCs, the plateau phase of muscarinic stimulated increase in $[\text{Ca}^{2+}]_i$ appeared to be quantitatively increased, while the magnitude of the initial peak to cholinergic agonists was not restored (Figure 4c and f). When TSMCs were incubated in Ca^{2+} -containing buffer and exposed to 50 nM ionomycin, there was a rapid increase of $[\text{Ca}^{2+}]_i$ (853 ± 160 nM, $n = 12$), followed by a steady plateau $[\text{Ca}^{2+}]_i$ which was sustained for several minutes (Figure 5). In the presence of 4 mM EGTA, the addition of ionomycin induced a rapid but only transient increase of $[\text{Ca}^{2+}]_i$ (703 ± 127 nM, $n = 14$). Addition of ACh or carbachol did not induce any further detectable increase in $[\text{Ca}^{2+}]_i$, no matter whether TSMCs were exposed to ionomycin in the absence or presence of extracellular Ca^{2+} (Figure 5). The data obtained from Figures 4 and 5 indicate that, whereas ionomycin and full muscarinic agonists (ACh and carbachol) both release intracellular pools of Ca^{2+} in TSMCs, the ionophore is capable of producing approximately twice the $[\text{Ca}^{2+}]_i$ response to ACh or carbachol.

IPs accumulation in intact and permeabilized TSMCs: dependence on calcium availability

When TSMCs were prelabelled with [^3H]-inositol for 2 days and then exposed to ACh or carbachol, a rapid release (5–30 s) of IP_3 , IP_2 and IP_1 occurred (Figure 6). Although

Table 2 The maximum increase in $[Ca^{2+}]_i$ induced by various muscarinic agonists

Agonist	Basal (nM)	Initial (nM)	Plateau (nM)	n
Acetylcholine	178 ± 46	501 ± 43 (323 ± 32)	390 ± 30 (212 ± 19)	7
Carbachol	202 ± 13	430 ± 36 (228 ± 18)*	378 ± 34 (176 ± 25)	10
Oxotremorine	213 ± 14	408 ± 12 (195 ± 21)*	346 ± 15 (133 ± 17)*	6
Bethanechol	233 ± 37	410 ± 23 (157 ± 29)*	369 ± 26 (113 ± 18)*	8
Arecoline	195 ± 24	381 ± 42 (186 ± 31)*	261 ± 33 (66 ± 10)*	5

To fura-2-loaded TSMCs was added either acetylcholine (100 μ M), carbachol (100 μ M), bethanechol (100 μ M), oxotremorine (1 mM) or arecoline (1 mM). Both initial peak and sustained plateau $[Ca^{2+}]_i$ values were monitored. The net increases in $[Ca^{2+}]_i$ above the basal level are shown in parentheses. Results shown are means $\pm$ s.e.mean for at least five separate experiments.

* $P < 0.01$, as compared with acetylcholine-induced responses.

Figure 4 Effect of EGTA on $[Ca^{2+}]_i$ levels induced by muscarinic agonist addition. (a), Fura-2-loaded TSMCs were exposed to acetylcholine (ACh) (100 μ M). The initial peak and plateau $[Ca^{2+}]_i$ values were 433 and 332 nM, respectively, whereas the resting $[Ca^{2+}]_i$ was 201 nM. (b) EGTA (4 mM) was added to TSMCs before the addition of ACh (extracellular $[Ca^{2+}]_o$ about 50 nM). The initial peak $[Ca^{2+}]_i$ (190 nM) is less than in (a); $[Ca^{2+}]_i$ returns to the resting level (129 nM) within 1 min. The addition of 10 μ M ionomycin (Iono) elicits a further rise in $[Ca^{2+}]_i$ to 646 nM. In (c) TSMCs were treated as in (b) but after the return of $[Ca^{2+}]_i$ to the resting value, 4 mM $CaCl_2$ (Ca^{2+}) was added and the $[Ca^{2+}]_i$ increased to 224 nM. The subsequent addition of 10 μ M atropine (Atr) reduced the $[Ca^{2+}]_i$ from 224 to 178 nM. (d–f) Parallel experiments were carried out using carbachol (CCh) as a muscarinic stimulant. (d), Fura-2-loaded TSMCs were exposed to carbachol (100 μ M). The initial peak and plateau $[Ca^{2+}]_i$ value were 413 and 387 nM, respectively, whereas the resting $[Ca^{2+}]_i$ was 189 nM. (e) To TSMCs was added 4 mM EGTA (extracellular $[Ca^{2+}]_o$ about 50 nM) before the addition of carbachol. The initial peak $[Ca^{2+}]_i$ obtained (171 nM) is less than in (d) and $[Ca^{2+}]_i$ returns to the resting level (117 nM) within 1 min. The addition of 10 μ M ionomycin (Iono) elicits a further rise in $[Ca^{2+}]_i$ to 484 nM. In (f) TSMCs were treated as in (e) but after the return of $[Ca^{2+}]_i$ to the resting value, 4 mM $CaCl_2$ (Ca^{2+}) was added and the $[Ca^{2+}]_i$ raised to 241 nM. Subsequent addition of 10 μ M atropine (Atr) reduced the $[Ca^{2+}]_i$ from 241 to 208 nM.

IP_3 was often the most rapidly released IP following the addition of agonist, the appearance of IP_3 and IP_2 were kinetically indistinguishable. Because total IPs accumulation was linear with time in the presence of 10 mM LiCl for at least 60 min, a 60 min incubation period was routinely employed in subsequent experiments. Without adding ACh or carbachol, only a limited amount of IPs was released from the prelabelled TSMCs during a 60 min incubation (data not shown). Addition of ACh (100 μ M), carbachol (100 μ M), oxotremorine (1 mM), bethanechol (1 mM), and arecoline (1 mM) increased the IPs accumulation by 13.8, 12.5, 3.8, 5.7 and 5.9 fold, respectively, over the control. Ionomycin ap-

peared to increase IPs accumulation 4 fold (Figure 7). However, treatment with 4 mM EGTA was found to block most of the increased IPs accumulation induced by the muscarinic agonists and the ionophore. Only very small increases in IPs accumulation were detected (Figure 7).

To assess more directly the role played by physiologically relevant Ca^{2+} concentrations in the regulation of the PLC activity, prelabelled TSMCs were first permeabilized in digitonin-containing KGEH buffer and then exposed to Ca^{2+} -EGTA buffer solutions. IPs accumulation was measured after 60 min incubation. Elevation of $[Ca^{2+}]_i$ from 2.7 to 617 nM resulted in a 1.6 fold increase of IPs accumulation, with an

Figure 5 The increase in $[Ca^{2+}]_i$ induced by addition of ionomycin (Iono) in the presence or absence of extracellular Ca^{2+} . (a) (1) Addition of 10 μ M ionomycin to TSMCs incubated in Ca^{2+} -containing buffer resulted in a rise in $[Ca^{2+}]_i$ that was sustained for several min. Addition of ACh (100 μ M) did not cause any further rise in $[Ca^{2+}]_i$. (2) TSMCs were incubated with Ca^{2+} -containing buffer and 4 mM EGTA for 5 min before the addition of ionomycin. Note that the transient rise in $[Ca^{2+}]_i$ and ACh failed to induce a further increase in $[Ca^{2+}]_i$. (b) Parallel experiments were performed using carbachol (CCh) as an agonist. (1) Addition of 10 μ M ionomycin to TSMCs incubated in Ca^{2+} -containing buffer, resulted in a rise in $[Ca^{2+}]_i$ that was sustained for several min. Addition of carbachol (100 μ M) did not cause any further rise in $[Ca^{2+}]_i$. (2) TSMCs were incubated with Ca^{2+} -containing buffer and 4 mM EGTA for 5 min before the addition of ionomycin. Note that the transient rise in $[Ca^{2+}]_i$ and carbachol failed to induce any further increase in $[Ca^{2+}]_i$.

EC_{50} of 35 nM ($n = 3$) (Figure 8). Addition of 50 μ M guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) further potentiated this Ca^{2+} -dependent accumulation of IPs with an EC_{50} for Ca^{2+} of approximately 11 nM (Figure 8). The effect of carbachol was also potentiated by the inclusion of 50 μ M GTP γ S. Under these conditions, the $[Ca^{2+}]_i$ required to elicit a 50% increase in IPs accumulation was reduced from 23 to 8 nM. When carbachol and GTP γ S were simultaneously present, the stimulated accumulation of IPs was $343 \pm 59\%$ of control ($n = 3$), a value that compares favourably with the degree of stimulation obtained in intact cells. Thus, GTP γ S and carbachol together elicited a synergistic increase in IPs accumulation. Both the effect of carbachol alone and the synergistic increase in IPs accumulation from permeabilized TSMCs could be blocked by the addition of atropine (Table 3). Taken together, these results indicate that stimulation of muscarinic receptors caused IPs accumulation in TSMCs through PLC activation which in turn was regulated by changes of physiologically relevant $[Ca^{2+}]_i$.

Figure 6 Rapid release of inositol phosphates from TSMCs in the presence of muscarinic agonist. Cells that had been prelabelled for 2 days with [3 H]-inositol were washed with KHS, and then incubated in the presence of either 100 μ M acetylcholine (ACh, $\bullet$), or 100 μ M carbachol (Δ). Reactions were terminated by the addition of 5% PCA and inositol phosphates were extracted and separated. Results shown are the means $\pm$ s.e. mean of triplicate determinations in three separate experiments. In the experiments shown, labelled IP $_3$ appeared before IP $_2$ which was kinetically indistinguishable from IP $_3$, but was always detected within 5–10 s after the addition of agonists.

Figure 7 Calcium-dependence of phosphatidylinositol 4,5-bisphosphate (PIP $_2$) breakdown in intact TSMCs. Cells were prelabelled with [3 H]-inositol for 2 days. Labelled cells were incubated in either KHS or KHS containing 4 mM EGTA. Then acetylcholine (100 μ M, 2), carbachol (100 μ M, 3), oxotremorine (1 mM, 4), bethanechol (1 mM, 5), arecoline (1 mM, 6), or ionomycin (10 μ M, 7), was added to appropriate wells (1 = basal). Reaction was terminated after a 60 min incubation and inositol phosphates were separated and quantitated. Results are expressed as mean $\pm$ s.e. mean from three separate experiments. * $P < 0.01$, as compared with ACh.

Figure 8 Increases in $[Ca^{2+}]_i$ release inositol phosphates (IPs) from digitonin-permeabilized TSMCs. $[^3H]$ -inositol-prelabelled cells were permeabilized and incubated in KGEH buffer with added Ca^{2+} to achieve the required $[Ca^{2+}]_i$ (as measured by the addition of fura-2). Cells were incubated for 60 min in the absence or presence of either carbachol ($\square$), GTP γ S ($\bullet$), or carbachol plus GTP γ S ($\circ$). The EC_{50} values of $[Ca^{2+}]_i$ for basal ($\blacksquare$), carbachol (100 μ M), GTP γ S (50 μ M), and carbachol plus GTP γ S were 35, 27, 11 and 8 nM, respectively. Results are expressed as means $\pm$ s.e.mean from three separate experiments determined in triplicate. $[Ca^{2+}]_i$ values of 0.37, 2.69, 7.93, 16.61, 39.89, 56.35, 100.4, 207.3 and 616.8 nM were obtained at Ca^{2+} /EGTA molar ratios of 0, 0.13, 0.25, 0.38, 0.50, 0.63, 0.75, 0.88 and 1, respectively, with EGTA maintained at a concentration of 2 mM.

Table 3 Effect of atropine on carbachol- and GTP γ S-induced inositol phosphates (IPs) accumulation in digitonin-permeabilized TSMCs

Treatment	IPs accumulation (d.p.m. $\times 10^{-4}$)
Basal	3.18 $\pm$ 0.27
Carbachol	5.18 $\pm$ 0.18
Atropine	3.09 $\pm$ 0.36
Atropine + carbachol	3.10 $\pm$ 0.05*
GTP γ S	6.64 $\pm$ 0.36
GTP γ S + carbachol	9.81 $\pm$ 0.06
Atropine + GTP γ S + carbachol	6.82 $\pm$ 0.09

Permeabilized TSMCs were incubated for 15 min in KGEH buffer and sufficient Ca^{2+} was added to provide a $[Ca^{2+}]_i$ of 616.8 nM, in the presence of either atropine (10 μ M), carbachol (100 μ M) or GTP γ S (50 μ M). The basal level represents the release of IPs obtained in the absence of agents. Results are expressed as mean $\pm$ s.e.mean from three separate experiments determined in triplicates.

* $P < 0.01$ as compared with carbachol-induced response.

Discussion

It is well established that muscarinic receptor-mediated increase of $[Ca^{2+}]_i$ is due to both Ca^{2+} release from internal stores and an influx from external sources (Berridge & Irvine, 1989). The present study demonstrated that IP₃ accumulation is regulated by Ca^{2+} , GTP γ S and muscarinic agonists in

digitonin-permeabilized TSMCs. These results suggest that: (1) both full and partial muscarinic agonists stimulate PIP₂ hydrolysis and increase $[Ca^{2+}]_i$ in TSMCs to different extents; (2) a GTP-binding protein participates in Ca^{2+} -induced IP₃ accumulation by PLC; and (3) Ca^{2+} and GTP γ S have a synergistic effect on IP₃ accumulation. The results obtained with fura-2 loaded TSMCs indicate that muscarinic agonists evoke a rapid increase in $[Ca^{2+}]_i$ to a peak value, followed by a slow decline and sustained $[Ca^{2+}]_i$. The increase is maintained as long as the muscarinic receptors are occupied by the agonist. These data are consistent with those obtained in swine tracheal smooth muscle (Shieh *et al.*, 1991) and in vascular smooth muscle (Sato *et al.*, 1988). Both the initial peak and the plateau phase were abolished by the muscarinic antagonist, atropine (Yang, 1992); thus these changes in $[Ca^{2+}]_i$ were due to the interaction of muscarinic agonists with muscarinic receptors.

The rapid initial increase in $[Ca^{2+}]_i$ due to the activation of muscarinic receptors by agonists has been shown to be mediated through the release of IP₃ and the subsequent mobilization of Ca^{2+} from internal stores (Berridge & Irvine, 1989). In the present study, the results obtained from the TSMCs indicate that the time course for IP₃ accumulation is correlated with the time of the peak rise in $[Ca^{2+}]_i$. Both partial and full muscarinic agonists for PIP₂ breakdown, mobilize Ca^{2+} from the same metabolic pools. The partial muscarinic agonists bethanechol, oxotremorine and arecoline produced relatively smaller increases in $[Ca^{2+}]_i$ than did the full agonists, ACh and carbachol. These results are consistent with previous studies demonstrating that muscarinic receptor agonists stimulate accumulation of different levels of IP₃ in tracheal smooth muscle (van Amsterdam *et al.*, 1989) and therefore increase $[Ca^{2+}]_i$ to different extents.

In agreement with others (Chew & Brown, 1986; Merrit & Rink, 1987; McDonough *et al.*, 1988; Yang, 1992), both the initial increase and the plateau of $[Ca^{2+}]_i$ were dependent on the availability of extracellular Ca^{2+} . Addition of EGTA abolished the plateau phase of $[Ca^{2+}]_i$. Although the rapid increase of $[Ca^{2+}]_i$ following muscarinic receptor activation in other types of cells was found not to be completely inhibited by a reduction of extracellular Ca^{2+} (Chew & Brown, 1986; Merrit & Rink, 1987; McDonough *et al.*, 1988; Yang, 1992) in the studies described here EGTA markedly reduced the initial $[Ca^{2+}]_i$ values in TSMCs. It is conceivable that the influx of extracellular Ca^{2+} plays an important role in the generation of the initial increase in $[Ca^{2+}]_i$.

It is also plausible that PLC activity is very sensitive to changes in $[Ca^{2+}]_i$ induced by addition of EGTA (Best, 1986). Based on our results, two lines of evidence support this suggestion. First, addition of EGTA reduced muscarinic-stimulated IP₃ accumulation. Second, the basal, and carbachol and GTP γ S-mediated IP₃ accumulation in digitonin-permeabilized TSMCs was regulated by physiologically relevant $[Ca^{2+}]_i$ concentrations. In the presence of carbachol and GTP γ S, a $[Ca^{2+}]_i$ of less than 10 nM was required for half-maximal activation of PIP₂ breakdown. Therefore, it is evident that a small reduction of $[Ca^{2+}]_i$ significantly retards PLC activation.

Further evidence for the role of Ca^{2+} availability in PIP₂ hydrolysis was obtained from experiments with ionomycin. The Ca^{2+} ionophore ionomycin is a pharmacological tool with which to evoke changes in $[Ca^{2+}]_i$ which bypass the receptor-operated mechanisms. Addition of ionomycin to intact TSMCs induced a 4 fold increase of IP₃ accumulation and a 4–5 fold increase in $[Ca^{2+}]_i$. Although the extent of ionomycin-stimulated IP₃ accumulation was only 30% of that induced by ACh, it is possible that the increase in $[Ca^{2+}]_i$ that follows muscarinic receptor activation makes some further contribution to PIP₂ hydrolysis due to the requirement of Ca^{2+} for maximal activation of PLC. This suggests that although the elevation of $[Ca^{2+}]_i$ influences PLC activity in TSMCs, the primary regulation is due to muscarinic activation of PLC.

One of the most notable features of stimulated PIP_2 hydrolysis in TSMCs is that muscarinic receptor-effector coupling in permeabilized cells is similar to that observed in intact cells in the presence of $\text{GTP}\gamma\text{S}$. In the presence of carbachol and $\text{GTP}\gamma\text{S}$, PIP_2 breakdown in permeabilized TSMCs appeared to be the same as that obtained in intact cells stimulated by carbachol alone. Since $\text{GTP}\gamma\text{S}$ alone could also stimulate IPs accumulation in permeabilized cells, a guanine nucleotide binding protein might be involved in the transduction process (Evans *et al.*, 1985; Merritt *et al.*, 1986; Jones *et al.*, 1988). Although carbachol could enhance IPs accumulation without the addition of $\text{GTP}\gamma\text{S}$, this ability presumably reflects the presence of residual endogenous guanine nucleotides still retained within the permeabilized cells. However, the combination of carbachol and $\text{GTP}\gamma\text{S}$ had a synergistic effect on IPs accumulation. $\text{GTP}\gamma\text{S}$ may play a role in the breakdown of PIP_2 that reduces the requirement of $[\text{Ca}^{2+}]_i$ for activation of PLC activity (Bradford & Rubin, 1986; Smith *et al.*, 1986). The availability of $[\text{Ca}^{2+}]_i$ ($> 30 \text{ nM}$) is required for stimulation of PIP_2 hydrolysis. A half-maximal (EC_{50}) increase of IPs accumulation induced by carbachol alone occurred at a $[\text{Ca}^{2+}]_i$ greater than 47 nM . In the presence of $\text{GTP}\gamma\text{S}$, only 11 nM $[\text{Ca}^{2+}]_i$ was required for carbachol to induce a half-maximal increase of IPs accumula-

tion. Therefore, guanine nucleotides appear to sensitize the transduction process such that a muscarinic agonist can effectively initiate PIP_2 hydrolysis at a $[\text{Ca}^{2+}]_i$ that is normally encountered in quiescent cells.

In conclusion, our results demonstrate that muscarinic receptor activation in TSMCs causes a rapid increase in $[\text{Ca}^{2+}]_i$ related to the extent of PIP_2 hydrolysis and followed by a sustained plateau $[\text{Ca}^{2+}]_i$. PLC activity in TSMCs appeared to be sensitive to small changes of $[\text{Ca}^{2+}]_i$. Consequently, PIP_2 hydrolysis, can be modulated by either a decrease or an increase of $[\text{Ca}^{2+}]_i$. Meanwhile, the results obtained from permeabilized TSMCs indicate that guanine nucleotides sensitize the transduction process, thereby enhancing carbachol-stimulated IPs accumulation at a $[\text{Ca}^{2+}]_i$ found in the cytoplasm of quiescent cells.

This work was supported by grants CMRP-340 and 273 from Chang Gung Medical Research Foundation and NSC82-0412-B182-007 from National Science Council, Taiwan. The authors are greatly indebted to Drs Anthony Herp and Hwa-Ming Hwang at Chang Gung Medical College for their critical reading of the manuscript and suggestions. Appreciation is also expressed to Dr Delon Wu for his encouragement.

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(Received January 26, 1993

Revised June 10, 1993

Accepted July 20, 1993)