Intracellular calcium in canine cultured tracheal smooth muscle cells is regulated by M₃ muscarinic receptors

¹Chuen Mao Yang, Ying-Ling Yo & Yen-Yi Wang

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

1 The regulation of cytosolic Ca²⁺ concentrations ([Ca²⁺]_i) during exposure to carbachol was measured directly in canine cultured tracheal smooth muscle cells (TSMCs) loaded with fura-2. Stimulation of muscarinic cholinceptors (muscarinic AChRs) by carbachol produced a dose-dependent rise in [Ca²⁺]_i which was followed by a stable plateau phase. The EC₅₀ values of carbachol for the peak and sustained plateau responses were 0.34 and 0.33 μM, respectively.

2 Atropine (10 μM) prevented all the responses to carbachol, and when added during a response to carbachol, significantly, but not completely decreased [Ca²⁺]_i within 5 s. Therefore, the changes in [Ca²⁺]_i by carbachol were mediated through the muscarinic AChRs.

3 AF-DX 116 (a selective M₂ antagonist) and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, a selective M₃ antagonist) inhibited the carbachol-stimulated increase in [Ca²⁺]_i with pK_B values of 6.4 and 9.4, respectively, corresponding to low affinity for AF-DX 116 and high affinity for 4-DAMP in antagonizing this response.

4 The plateau elevation of [Ca²⁺]_i was dependent on the presence of external Ca²⁺. Removal of Ca²⁺ by the addition of 2 mM EGTA caused the [Ca²⁺]_i to decline rapidly to the resting level. In the absence of external Ca²⁺, only an initial transient peak of [Ca²⁺]_i was seen which then declined to the resting level; the sustained elevation of [Ca²⁺]_i could then be evoked by the addition of Ca²⁺ (1.8 mM) in the continued presence of carbachol.

5 Ca²⁺ influx was required for the changes of [Ca²⁺]_i, since the Ca²⁺-channel blockers, diltiazem (10 μM), nifedipine (10 μM), verapamil (10 μM) and Ni²⁺ (5 mM), decreased both the initial and sustained elevation of [Ca²⁺]_i in response to carbachol. These Ca²⁺-channel blockers also decreased the sustained elevation of [Ca²⁺]_i when applied during the plateau phase.

6 In conclusion, we have demonstrated that the initial detectable increase in carbachol-stimulated [Ca²⁺]_i is due to the release of Ca²⁺ from internal stores, followed by the flux of external Ca²⁺ into the cells. This influx of extracellular Ca²⁺ partially involves an L-type Ca²⁺-channel. M₃ muscarinic receptors appear to mediate the Ca²⁺ mobilization in canine TSMCs.

Keywords: Tracheal smooth muscle cells; Ca²⁺; muscarinic receptors; Ca²⁺-channel blockers

Introduction

Stimulation of muscarinic cholinceptor (muscarinic AChRs) by agonists evokes a contractile response in several smooth muscles. The mechanism of stimulus-response coupling has been extensively studied in tracheal smooth muscle (Farley & Miles, 1978; Park & Rasmussen, 1986; Grandordy *et al.*, 1986; Gunst & Bandyopadhyay, 1989; Yang *et al.*, 1991a). In the trachea, stimulation of muscarinic AChRs activates phospholipase C, via a cholera toxin-sensitive G-protein (G_p) (Yang *et al.*, 1991a), which hydrolyzes phosphoinositide, leading to the formation of inositol-1,4,5-triphosphate (IP₃) (Baron *et al.*, 1984; Grandordy *et al.*, 1986; Meurs *et al.*, 1988; Chilvers *et al.*, 1989; Roffel *et al.*, 1990; Yang *et al.*, 1991a,b). This in turn stimulates the release of Ca²⁺ from its internal stores, triggering contraction of tracheal smooth muscle (Hashimoto *et al.*, 1985). Therefore, IP₃-mediated Ca²⁺ release appears to be an important fact in the contractile response, particularly during the developmental stage of force in the tracheal smooth muscle. There is a high metabolic flux of phosphoinositide during contraction (Baron *et al.*, 1989). Muscarinic agonists induce increases in intracellular Ca²⁺ ([Ca²⁺]_i) concentration and in force in the tracheal smooth muscle under Ca²⁺-free conditions (Takuwa *et al.*, 1987), but muscarinic-induced contractions are markedly slower to develop under conditions where phosphoinositide hydrolysis and IP₃ accumulation are inhibited by phorbol ester (Baba *et al.*, 1989).

In contrast, entry of Ca²⁺ from the external environment into the vascular smooth muscle during agonist stimulation plays an important role in maintaining the plateau phase of a prolonged contraction. This plateau phase disappears in a Ca²⁺-free medium (Cauvin & Van Breemen, 1985). Other studies also indicate that the initial increase of [Ca²⁺]_i is indeed transient, but that the decline does not fall to the resting level in the presence of external Ca²⁺ (Merritt & Rink, 1987; Shieh *et al.*, 1991). Therefore, other mechanisms may operate during the prolonged response. Putney (1986) proposed that the two phases of increase in [Ca²⁺]_i may be intimately linked: IP₃ first stimulates the release of Ca²⁺ from the internal stores and the depletion of these stores then becomes the signal for the entry of Ca²⁺ from outside the cell.

In our laboratory, both M₂ and M₃ receptor subtypes have been shown to exist in canine TSMCs (Yang, 1991) that are coupled to the inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation and to inositol phosphate (IP) accumulation, respectively (Yang *et al.*, 1991a). Furthermore, the M₃ receptor subtype has been demonstrated to couple to Ca²⁺ mobilization in SH-SY5Y and Lan-1 human neuroblastoma cells (Murphy *et al.*, 1991; Fatatis *et al.*, 1992). Therefore, these experiments were carried out to determine whether activation of M₃ receptors in canine TSMCs results in an increase of [Ca²⁺]_i.

Defining the cellular mechanisms that mediate a prolonged muscle contraction is important in understanding tracheal smooth muscle function; therefore we have undertaken these

¹ Author for correspondence.

studies to clarify in part the nature of changes in $[Ca^{2+}]_i$ during continued exposure to carbachol. The purpose of the present study was two fold: to investigate the changes in $[Ca^{2+}]_i$ during exposure to carbachol and to assess the contribution of Ca^{2+} from various sources to these changes in $[Ca^{2+}]_i$ in cultured TSMCs. The results show that the changes in $[Ca^{2+}]_i$ during the occupation of muscarinic AChRs by carbachol are due to an immediate, but transient release of Ca^{2+} from the internal stores as well as to the entry of Ca^{2+} from outside the cells, persisting as long as the muscarinic AChRs are continuously exposed to carbachol. The extent of Ca^{2+} influx through Ca^{2+} -channels, depends, in part, on the Ca^{2+} concentration outside the cells. Furthermore, we have pharmacological evidence that the muscarinic AChR subtype which is coupled to Ca^{2+} mobilization is the M_3 receptor type in canine TSMCs.

Methods

Animals

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

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to the methods previously reported (Yang, 1991; 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 the dissociation medium containing 0.1% collagenase IV, 0.025% DNase I, 0.025% elastase IV, and antibiotics in physiological solution. The physiological solution contained (mM): NaCl 137, KCl 5, CaCl₂ 1.1, NaHCO₃ 20, NaH₂PO₄ 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 residual material 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% FBS, were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts. The cells were counted and diluted with DMEM/F-12 to a final concentration of 5×10^5 cells ml⁻¹. The cells (2 ml/well) were plated onto 6-well culture plates containing glass coverslips coated with collagen. The medium was changed after 24 h and then every 3 days. After 5 days in culture, the 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 µg ml⁻¹) for 12–14 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts the cells were identified by an indirect immunofluorescence method (Gown *et al.*, 1985). Over 95% of the cells were smooth muscle cells.

Measurement of intracellular Ca^{2+} levels

$[Ca^{2+}]_i$ was measured in confluent 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 for one day before measurements were taken. The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 µM fura-2/AM and were incubated at 37°C for 45 min. At the end of the loading

period, the coverslips were washed twice with the physiological buffer solution (PBS) containing (mM): NaCl 125, KCl 5, CaCl₂ 1.8, MgCl₂ 2, NaH₂PO₄ 0.5, NaHCO₃ 5, HEPES 10 and glucose 10, pH 7.4. The cells were then incubated in PBS for another 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 temperature controlled holder of a SLM 8000C spectrofluorometer. Continuous stirring was achieved with a magnetic stirrer.

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 emission wavelength 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) to the fluorescence at the two wavelengths was 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 µM) 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

The EC₅₀ of carbachol for stimulating $[Ca^{2+}]_i$ was estimated by Graph Pad programme (Graph Pad, San Diego, California, U.S.A.). The dissociation constants (K_B) of muscarinic antagonists were estimated by the method of Furchgott (1972), from their ability to antagonize carbachol-mediated increase in $[Ca^{2+}]_i$.

The data are expressed as the mean $\pm$ s.e.mean of at least four experiments with statistical comparisons based on a two-tailed Student's paired *t* test at a $P < 0.01$ 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 (Germany). Fura-2/AM was ordered from Molecular Probes Inc (Eugene, OR, U.S.A.). AF-DX 116 (11-[(2-[(diethylamino)methyl]-1-piperidinyl) acetyl]-5, 11-dihydro-6H-pyrido [2,3-b] [1,4]benzodiazepine-6-one) was a gift from Dr K. Noll at Dr Karl Thomae, Germany. 4-Diphenylacetoxy-N-methylpiperidine (4-DAMP) was purchased from Research Biochemicals Inc (Natick, MA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St Louis, MO, U.S.A.).

Results

Effects of carbachol on $[Ca^{2+}]_i$

Stimulation of muscarinic AChRs by carbachol (100 µM) rapidly generated a peak and then a sustained increase in $[Ca^{2+}]_i$, as measured directly in cultured canine TSMCs loaded with fura-2 in the presence of external Ca^{2+} (Figure 1). The calculated resting value of $[Ca^{2+}]_i$ was 105 ± 28 nM ($n = 36$). The initial transient $[Ca^{2+}]_i$ peak (318 ± 25 nM, $n = 18$) was reached within 30 s and was followed by a sustained elevation of $[Ca^{2+}]_i$ for at least 5 min in the presence of carbachol. During prolonged carbachol application, $[Ca^{2+}]_i$ was significantly higher (282 ± 29 nM, $n = 18$, $P < 0.001$) than its resting value. When the potent muscarinic antagonist, atropine (10 µM), was added during the plateau phase, the $[Ca^{2+}]_i$ decreased to 165 ± 30 nM ($n = 20$) within 60 s. This level is substantially higher than that of the resting level ($P < 0.01$). Both the peak and plateau of $[Ca^{2+}]_i$ in-

Figure 1 Effect of carbachol on $[Ca^{2+}]_i$ in canine tracheal smooth muscle cells (TSMCs). Confluent cultures of canine TSMCs on glass coverslips were loaded with 5 μ M fura-2 and fluorescent measurement of $[Ca^{2+}]_i$ was carried out in a dual excitation wavelength spectrofluorometer, with excitation at 340 and 380 nm. Trace (a) cell cultures were added with carbachol (100 μ M) at 5 min after incubation. The initial transient $[Ca^{2+}]_i$ rose from 95 nm to 298 nm in 30 s. This level was sustained with only a slight decline during the presence of carbachol. After 5 min of carbachol exposure, atropine (10 μ M) was added, whereupon the $[Ca^{2+}]_i$ decreased from the plateau level to 165 nm within 60 s. Trace (b) atropine (10 μ M) was added at the beginning. After 5 min incubation, carbachol (100 μ M) was added, no change in $[Ca^{2+}]_i$ was seen.

Figure 2 Competitive antagonism of carbachol-mediated increase in $[Ca^{2+}]_i$ by (a) control, (b) AF-DX 116, and (c) 4-DAMP. Tracheal smooth muscle cells (TSMCs) were incubated in the presence of AF-DX 116 (10 μ M) or 4-DAMP (10 nM) for 5 min before the addition of increasing concentrations of carbachol as indicated for each trace. For abbreviations, see text.

crease were abolished when atropine was added before carbachol, suggesting that the increase of $[Ca^{2+}]_i$ after carbachol exposure required occupation of muscarinic AChRs.

Effect of muscarinic antagonists on $[Ca^{2+}]_i$

The carbachol-stimulated effect on $[Ca^{2+}]_i$ was dose-dependent (Figure 2). The biphasic responses became less pronounced as the carbachol concentration was reduced (Figure 2). The half-maximal effect of carbachol (EC_{50}) for the initial peak was 0.33 μ M (Figure 3) and 0.34 μ M for the sustained phase.

In order to differentiate the muscarinic AChR subtype that is coupled to changes in $[Ca^{2+}]_i$, we compared the affinity of selective M₂ and M₃ antagonists, AF-DX 116 and 4-DAMP, on the inhibition of carbachol-induced increase in $[Ca^{2+}]_i$. As shown in Figure 2, preincubation of fura-2-loaded TSMCs with these antagonists inhibited both the initial peak and the sustained phase of changes in $[Ca^{2+}]_i$. The concentration-effect relationship of carbachol was shifted to the right in a nearly parallel fashion, upon addition of AF-DX 116 (10 μ M) and 4-DAMP (10 nM) (see Figure 3). The EC_{50} values of carbachol were increased from 0.33 μ M to 12 and 16 μ M in the presence of AF-DX 116 and 4-DAMP, respectively. The pK_B values of AF-DX 116 and 4-DAMP for antagonizing the carbachol-induced $[Ca^{2+}]_i$ were 6.4 and 9.4, respectively, which were in good agreement with the low affinity for AF-DX 116 and high affinity for 4-DAMP in this response (see review, Hulme *et al.*, 1990).

Figure 3 The concentration-response curves of carbachol for the increase in $[Ca^{2+}]_i$ by (●) control, (○) AF-DX 116, 10 μ M, and (△) 4-DAMP, 10 nM. Each point represents the mean $\pm$ s.e.mean of ten measurements.

Effect of external Ca^{2+}

To define the mechanisms of the changes in the initial and sustained elevation of $[\text{Ca}^{2+}]_i$ in response to carbachol, experiments were carried out to examine the changes in $[\text{Ca}^{2+}]_i$ induced by carbachol with or without external Ca^{2+} . As shown in Figure 4. Trace a indicates the increase in $[\text{Ca}^{2+}]_i$ when TSMCs were exposed to carbachol (100 μM) in the presence of external Ca^{2+} (1.8 mM). As soon as carbachol was added, an immediate increase in $[\text{Ca}^{2+}]_i$ was seen and reached a peak (297 ± 35 nM, $n = 9$) within a few seconds. The peak was followed by a short, but sharp decline (249 ± 42 nM, $n = 9$) which became gradual during the continuous exposure to carbachol. Removal of the external Ca^{2+} by the addition of 2 mM EGTA during the sustained phase of $[\text{Ca}^{2+}]_i$ caused an abrupt decline to its resting level within 10 s. Trace b represents the response of TSMCs exposed to carbachol in the absence of external Ca^{2+} . The profile shows an immediate transient increase in $[\text{Ca}^{2+}]_i$ similar to trace a, but of a lesser magnitude (197 ± 17 nM, $n = 9$). The peak rapidly decayed within 10 s to the resting level. There was no sustained elevation of $[\text{Ca}^{2+}]_i$ in the absence of external Ca^{2+} during exposure to carbachol. When Ca^{2+} (1.8 mM) was added 10 min after the addition of carbachol to the solution, an immediate and sustained increase in $[\text{Ca}^{2+}]_i$ (255 ± 30 nM, $n = 9$) was observed, reaching a level equal to the sustained elevation of $[\text{Ca}^{2+}]_i$ in TSMCs of trace a. These results suggest that the changes in $[\text{Ca}^{2+}]_i$ caused by carbachol in canine TSMCs required two sources of Ca^{2+} : (1) stored intracellular Ca^{2+} that is immediately but transiently mobilized upon receptor occupation; and (2) an influx of external Ca^{2+} into the cells that accounts for the sustained high level of $[\text{Ca}^{2+}]_i$. These processes occur simultaneously but may also be separated under appropriate conditions, as shown in Figure 4.

Because the sustained increase of $[\text{Ca}^{2+}]_i$ required external Ca^{2+} , we explored this effect as a function of the external Ca^{2+} concentration. Typical results are shown in Figure 5, where TSMCs were exposed to carbachol in the presence of 1.8 (trace a), 1.0 (trace b), 0.2 (trace c) and 0 (Ca^{2+} -free; trace d) mM Ca^{2+} , respectively.

Figure 4 Effect of external Ca^{2+} on carbachol-stimulated changes in $[\text{Ca}^{2+}]_i$. Trace (a): cells were stimulated by adding carbachol (100 μM) to the buffer containing 1.8 mM Ca^{2+} . An immediate increase in $[\text{Ca}^{2+}]_i$ was seen which was followed by a sustained plateau level of $[\text{Ca}^{2+}]_i$. The plateau level decreased sharply when 2 mM EGTA was added during the sustained plateau level in the presence of carbachol. Trace (b): cells were incubated in the absence of external Ca^{2+} and stimulated by carbachol (100 μM). The profile shows an initial transient increase of $[\text{Ca}^{2+}]_i$ similar to trace (a) but to a substantially smaller degree. The sustained plateau $[\text{Ca}^{2+}]_i$ was not seen in the absence of the external Ca^{2+} . However, when Ca^{2+} (1.8 mM) was added 10 min after the addition of carbachol, a sustained increase of $[\text{Ca}^{2+}]_i$ occurred, reaching a level equal to the sustained elevation of $[\text{Ca}^{2+}]_i$ in tracheal smooth muscle cells observed in trace (a).

Figure 5 Carbachol-induced changes in $[\text{Ca}^{2+}]_i$ in tracheal smooth muscle cells (TSMCs) vary with external Ca^{2+} concentrations. Traces were obtained when TSMCs were stimulated with carbachol (100 μM) in the presence of external Ca^{2+} equal to 1.8 mM (trace a), 1.0 mM (trace b), 0.2 mM (trace c), or Ca^{2+} -free (trace d). All traces show a similar trend except for the changes in $[\text{Ca}^{2+}]_i$ levels which are dependent on the external concentrations.

trace d) mM Ca^{2+} , respectively. All traces showed a similar trend, although the level was dependent on the external Ca^{2+} concentration. These results suggest that Ca^{2+} influx during the occupation of muscarinic AChRs is both immediate and persistent.

Effects of calcium-channel blockers on $[\text{Ca}^{2+}]_i$

Such a sustained elevation in $[\text{Ca}^{2+}]_i$ probably involves Ca^{2+} -channels which may be operated by depolarization (Takuwa *et al.*, 1987). We therefore assessed the effect of Ca^{2+} -channel blockers on the carbachol-stimulated changes in $[\text{Ca}^{2+}]_i$. The Ca^{2+} -channel antagonists, diltiazem, nifedipine, and verapamil, were added either before or after carbachol (Table 1). The carbachol-stimulated initial transient increase of $[\text{Ca}^{2+}]_i$ was significantly reduced by diltiazem (10 μM), nifedipine (10 μM), or verapamil (10 μM). The initial transient increase of $[\text{Ca}^{2+}]_i$ decreased from 318 ± 25 nM in the control to 226 ± 18 nM with diltiazem, to 180 ± 23 nM with nifedipine, and to 232 ± 20 nM with verapamil ($n = 9$), as well as decreased the plateau phase of $[\text{Ca}^{2+}]_i$ from 282 ± 29 nM to 196 ± 15 , 151 ± 17 , and 185 ± 17 nM, ($n = 9$), respectively, when the Ca^{2+} -channel blockers were applied before the addition of carbachol. When diltiazem, nifedipine, or verapamil was added to the solution during the sustained plateau

Table 1 Effects of diltiazem, nifedipine, verapamil, and Ni^{2+} on carbachol-stimulated changes in $[\text{Ca}^{2+}]_i$

Drug	Carbachol/Blocker Peak	Blocker/Carbachol Plateau	Blocker/Carbachol Peak	Blocker/Carbachol Plateau
Control	318 ± 25	282 ± 29		
Diltiazem	315 ± 16	248 ± 13	$226 \pm 18^*$	$196 \pm 15^*$
Nifedipine	323 ± 19	$184 \pm 19^*$	$180 \pm 23^*$	$151 \pm 17^*$
Verapamil	302 ± 21	$192 \pm 18^*$	$232 \pm 20^*$	$185 \pm 17^*$
Ni^{2+}	295 ± 32	$153 \pm 11^*$	$188 \pm 15^*$	$151 \pm 16^*$

TSMCs were incubated in the presence of external Ca^{2+} (1.8 mM). Diltiazem (10 μM), nifedipine (10 μM), verapamil (10 μM) and Ni^{2+} (5 mM) were added to the buffer before (Blocker/Carbachol) or during response (Carbachol/Blocker) to carbachol (100 μM). Data are expressed as concentration of $[\text{Ca}^{2+}]_i$ in nM.

* $P < 0.01$ as compared with non-treated cells induced by carbachol.

phase, the sustained elevation of $[Ca^{2+}]_i$ decreased from 282 ± 29 nM to 248 ± 13 nM with diltiazem, to 185 ± 19 nM with nifedipine, and to 192 ± 18 nM with verapamil ($n = 9$). Ni^{2+} (5 mM), a transition metal ion that blocks voltage-gated Ca^{2+} -channels, also decreased the initial transient $[Ca^{2+}]_i$ from 318 ± 25 to 188 ± 15 nM ($n = 11$) as well as the sustained elevation of $[Ca^{2+}]_i$ from 282 ± 29 to 151 ± 11 nM ($n = 11$) (Table 1), when added before carbachol. Addition of 5 mM Ni^{2+} during the plateau phase abolished the sustained $[Ca^{2+}]_i$ level evoked by carbachol. If Ni^{2+} was added before carbachol, the response resembled that seen in a Ca^{2+} -free solution in Figure 4 (Table 1).

Discussion

Smooth muscle contraction is initiated by agonists which bind to a receptor and induce an increase in $[Ca^{2+}]_i$. Calcium release from internal stores and influx from external sources play an important role in the regulation of $[Ca^{2+}]_i$ (Bolton, 1979; Van Breeman & Saida, 1989). The present study demonstrates that muscarinic AChR-mediated increase in $[Ca^{2+}]_i$ is also due to calcium release from the internal stores and to an influx from the external source. Although both influx and intracellular release of calcium appear to be initiated simultaneously, intracellular release triggers a rapid peak in $[Ca^{2+}]_i$ which is transient even in the continued presence of carbachol. In contrast, the influx component of $[Ca^{2+}]_i$ reaches its maximum more slowly, is of lower magnitude, and persists as long as carbachol is present. These changes in $[Ca^{2+}]_i$ were directly determined in cultured canine TSMCs by use of the calcium-sensitive dye, fura-2.

Carbachol produced a dose-dependent, biphasic increase in $[Ca^{2+}]_i$ which displayed an initial transient peak and a sustained plateau phase. However, the peak is only apparent at higher concentrations of carbachol. The differences in Ca^{2+} mobilization can be due to varying extents of receptor occupancy by muscarinic agonists (Al-Hassani *et al.*, 1993). Thus low concentrations of agonists may not be potent enough to stimulate the release of $[Ca^{2+}]_i$, leaving extracellular Ca^{2+} influx as the primary source of Ca^{2+} for activation of tracheal smooth muscle contraction. Both the initial peak and the plateau phase were prevented by the addition of the muscarinic antagonist, atropine. However, once $[Ca^{2+}]_i$ is initiated by carbachol, even atropine did not completely reverse the effect (Figure 1), while depletion of external Ca^{2+} did (Figure 4). Thus it appears that some event is initiated by carbachol and is continuing to occur in the presence of atropine. This event still involves an influx of extracellular Ca^{2+} since EGTA completely abolishes this response. This clearly indicates that these changes in $[Ca^{2+}]_i$ were due to the association of carbachol with specific muscarinic AChRs.

Our results demonstrate that the muscarinic AChRs coupled to $[Ca^{2+}]_i$ have a low affinity for AF-DX 116 ($pK_B = 6.4$) and high affinity for 4-DAMP ($pK_B = 9.4$). Therefore, they can be classified as M₃ muscarinic receptors (Hulme *et al.*, 1990). These results are consistent with an M₃ receptor being linked to the rise in $[Ca^{2+}]_i$ (Murphy *et al.*, 1991) and are in good agreement with our previous report that activation of M₃ muscarinic receptors leads to generation of IP₃ and muscle contraction (Yang *et al.*, 1991a).

In the absence of external Ca^{2+} , carbachol produced a rapid increase in $[Ca^{2+}]_i$ which declined to the resting level within 10 s. An initial transient elevation of $[Ca^{2+}]_i$ still occurred in the absence of any external Ca^{2+} but to a substantially smaller extent than that measured in the presence of external Ca^{2+} . This smaller elevation of $[Ca^{2+}]_i$ can be taken as representative of the IP₃-mediated release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1989). In tracheal smooth muscle, it is known that stimulation of muscarinic AChRs by agonists results in the activation of phospholipase C which hydrolyzes phosphoinositide and leads to the formation of IP₃ (Baron *et al.*, 1984; Grandordy

et al., 1986; Meurs *et al.*, 1988; Chilvers *et al.*, 1989; Roffel *et al.*, 1990; Yang *et al.*, 1991a,b). Carbachol-stimulated IP₃ accumulation was maximized occurring within 5 to 10 s and then fell to or below the basal levels within 60 s. This profile is very similar to the time course described for the initial transient $[Ca^{2+}]_i$ induced by carbachol in canine TSMCs.

An important observation was that the addition of 1.8 mM external Ca^{2+} to the Ca^{2+} -free medium rapidly brought $[Ca^{2+}]_i$ to the sustained plateau level after $[Ca^{2+}]_i$ had returned to the resting level as the cells continued to be exposed to carbachol. It appears that muscarinic AChR occupation by carbachol and the presence of external Ca^{2+} are necessary and sufficient conditions for Ca^{2+} influx. However, the mechanism regulating this influx was obscure. Our data provide evidence that the two phases of the carbachol-stimulated increase in $[Ca^{2+}]_i$ are mediated by two different mechanisms. An increase in intracellular Ca^{2+} concentration is generally caused by its release from internal stores or by entry through the cell membrane from an external source (Bolton, 1979; Van Breemen & Saida, 1989). Calcium entry through the cell membrane can be mediated via voltage- and receptor-operated calcium channels. The initial transient $[Ca^{2+}]_i$ has been shown to be dependent on both internal and external Ca^{2+} since it was attenuated but not abolished by calcium channel blockers. These organic Ca^{2+} -channel blockers did not attenuate the rise in $[Ca^{2+}]_i$ induced by carbachol at a concentration of 1 μ M, though at a 30 μ M concentration they caused a reduction of both the peak and plateau phase of $[Ca^{2+}]_i$ (Fatatis *et al.*, 1992). In this study, diltiazem, nifedipine, and verapamil only partially attenuated and Ni^{2+} completely reversed the plateau phase at a concentration of 10 μ M, when they were applied after the initiation of $[Ca^{2+}]_i$ response by carbachol. Not only Ni^{2+} , but all other Ca^{2+} -channel antagonists inhibited the plateau phase of $[Ca^{2+}]_i$, when these antagonists were added before the addition of carbachol. This indicates that the sustained plateau of $[Ca^{2+}]_i$ is mediated by Ca^{2+} influx through those L-type calcium channels which are sensitive to calcium channel blockers. These changes in $[Ca^{2+}]_i$ implicated in the regulation of smooth muscle contraction have been demonstrated (Bolton, 1979; Takuwa *et al.*, 1987; Baba *et al.*, 1989; Al-Hassani *et al.*, 1993). It has been suggested that the initial rise in $[Ca^{2+}]_i$ which occurs during force development is due to the release of intracellular Ca^{2+} , whereas the plateau phase of $[Ca^{2+}]_i$ and of tension in tracheal smooth muscle result from an interplay between increases in the influx of extracellular Ca^{2+} and the release of Ca^{2+} from internal stores (Takuwa *et al.*, 1987).

A number of other characteristics of the sustained elevation of $[Ca^{2+}]_i$ were observed. Ni^{2+} appears to block both voltage-sensitive Ca^{2+} channels (Tsien, 1983) and receptor-operated Ca^{2+} entry in platelets (Hallam & Rink, 1985). When Ni^{2+} is added before carbachol, it is able to abrogate the sustained plateau of $[Ca^{2+}]_i$, but the initial transient $[Ca^{2+}]_i$ is not completely abolished. Addition of Ni^{2+} during the sustained plateau of $[Ca^{2+}]_i$ evoked by carbachol caused the $[Ca^{2+}]_i$ to decline immediately to the resting level and was consistent with blockage of Ca^{2+} entry (Tsien, 1983).

In conclusion, our data show that muscarinic receptor-stimulated canine tracheal smooth muscle contraction is mediated by Ca^{2+} release from the internal stores and by the entry of extracellular Ca^{2+} through calcium channels. These results suggest that the initial peak results from IP₃-mediated Ca^{2+} release from intracellular stores and that sustained elevation of $[Ca^{2+}]_i$ is due to an agonist-dependent influx of Ca^{2+} from external sources. These changes in $[Ca^{2+}]_i$ relate to the generation of tension in the canine tracheal smooth muscle (Takuwa *et al.*, 1987; Al-Hassani *et al.*, 1993).

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