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$[Ca^{2+}]_i$ oscillations induced by muscarinic stimulation in airway smooth muscle cells: receptor subtypes and correlation with the mechanical activity

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1 Cytosolic calcium concentration ($[Ca^{2+}]_i$) by indo 1 microspectrofluorimetry in freshly isolated cells and isometric contraction of isolated rings were measured in response to muscarinic cholinoceptor stimulation in rat tracheal smooth muscle.

2 In isolated myocytes, acetylcholine (ACh, 0.03–1 μM) caused a rapid and graded increase in $[Ca^{2+}]_i$ up to a net amplitude of 492 ± 26 nM ($n = 19$) which gradually declined. The EC_{50} for ACh was 0.13 μM. This first $[Ca^{2+}]_i$ peak was followed, when the ACh concentration increased, in approximately 50–60% of the cells, by successive peaks of decreased amplitude ($[Ca^{2+}]_i$ oscillations) superimposed on the plateau phase. Whereas the percentage of cells exhibiting $[Ca^{2+}]_i$ oscillations remained consistent, the frequency of these oscillations increased to up to 10 min^{-1} with an ACh concentration of 100 μM.

3 Removal of extracellular calcium (in the presence of EGTA, 0.4 mM) or addition of the voltage-dependent Ca^{2+}-channel blocker verapamil (10 μM) did not alter the first $[Ca^{2+}]_i$ peak, the plateau or the oscillations induced by ACh or carbachol. In contrast, the specific inhibitor of the sarcoplasmic Ca^{2+}-ATPase, thapsigargin (1 μM), completely abolished the $[Ca^{2+}]_i$ response. Thapsigargin (1 μM) also blocked the caffeine (5 mM)-induced transient rise in $[Ca^{2+}]_i$.

4 Atropine (a non-selective muscarinic cholinoceptor antagonist) and 4-diphenyl acetoxy N-methyl piperidine (4-DAMP, a selective M_3 antagonist) inhibited the $[Ca^{2+}]_i$ response to muscarinic cholinoceptor activation with an IC_{50} of 13 and 20 nM, respectively. Pirenzepine (a selective M_1 antagonist) also totally inhibited the $[Ca^{2+}]_i$ response to ACh but with a higher IC_{50} of 2 μM. Methoctramine (a selective M_2 antagonist) up to a concentration of 10 μM caused only a 40% inhibition. The effect of muscarinic antagonists on cumulative concentration-response curves (CCRC) for carbachol was assessed at the following concentrations: atropine and 4-DAMP at 3, 10 and 30 nM; pirenzepine 0.3, 1 and 3 μM, and methoctramine at 1, 3 and 10 μM. For these concentrations, all of the antagonists produced a rightward shift of the CCRC for carbachol and pA_2 values were 9.2, 8.8, 6.7 and 6.3, respectively.

5 In conclusion, the present study indicates that muscarinic stimulation of rat isolated tracheal smooth muscle cells induces $[Ca^{2+}]_i$ oscillations. The occurrence of these oscillations depends on the graded amplitude of the first $[Ca^{2+}]_i$ rise and their frequency may play a role in the amplitude of the mechanical activity in response to muscarinic cholinoceptor activation. Both the $[Ca^{2+}]_i$ and the contractile responses are primarily dependent on activation of the M_3 receptor subtype.

Keywords: Smooth muscle; cytosolic calcium; excitation-contraction coupling; rat trachea; airways; calcium oscillations; muscarinic cholinoceptor subtypes; acetylcholine; freshly isolated myocytes; indo 1

Introduction

Stimulation of muscarinic cholinoceptors is a major determinant of airway smooth muscle tone. Cholinomimetic agents are among the most efficacious agonists of airway smooth muscle contraction in a variety of species including man and at a very early stage in the course of ontogenesis (Fayon *et al*., 1994). Although muscarinic-induced contraction in airway smooth muscle has been shown to be largely independent of the extracellular calcium source in all species including man (Farley & Miles, 1978; Marthan *et al*., 1987), calcium sources and pathways in response to muscarinic cholinoceptor stimulation are currently more precisely re-investigated in isolated airway smooth muscle cells by use of fluorescent dyes.

In cultured airway smooth muscle cells, muscarinic as well as other agonists usually produce a biphasic response in $[Ca^{2+}]_i$ (Murray & Kotlikoff, 1991; Yang *et al*., 1991; 1993a,b; Marsh & Hill, 1993; Amrani *et al*., 1995; Tolloczko *et al*., 1995). The transient first Ca^{2+} peak is ascribed to the mobilization of intracellular Ca^{2+} stores by inositol 1,4,5-trisphosphate (IP_3), the Ca^{2+} releasing second messenger coupled to muscarinic stimulation (Roffel *et al*., 1990; Yang *et al*., 1991; Al-Hassani *et al*., 1993). The mechanism underlying the secondary, steady-state phase of the response remains unclear, and the role of extracellular calcium is controversial. In particular, the extent to which an influx of extracellular calcium through L-type calcium channel contributes to this steady-state phase is a matter of debate (Murray & Kotlikoff, 1991; Yang *et al*., 1993a,b).

Recently another pattern of agonist-induced $[Ca^{2+}]_i$ responses, the so-called $[Ca^{2+}]_i$ oscillations, initially described in non-excitable cells (Berridge, 1993), has been observed in smooth muscle cells. As a general rule, these oscillations have been described in freshly isolated smooth muscle cells. $[Ca^{2+}]_i$ oscillations can arise from entry of Ca^{2+} across the plasma membrane through voltage-gated Ca^{2+} channels associated with membrane depolarization (membrane oscillator) and/or from release and re-uptake of Ca^{2+} in intracellular stores (cytosolic oscillator). In the pulmonary arterial myocyte we observed $[Ca^{2+}]_i$ oscillations in response to angiotensin II, an agonist inducing a primarily inositol triphosphate (IP_3)-dependent Ca^{2+} release (Guibert *et al*., 1996). A combination of two types of $[Ca^{2+}]_i$ oscillations (membrane and cytosolic oscillators) have been observed in response to carbachol in single smooth muscle cells of guinea-pigs ileum (Kohda *et al*., 1996).

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In porcine tracheal smooth muscle cells, by using the amplitude of the mean inward calcium activated chloride current as an index of the amplitude of $[Ca^{2+}]_i$ rise and the fluorescent dye fluo-3, Liu & Farley (1996a,b) recently described $[Ca^{2+}]_i$ oscillations in response to acetylcholine the occurrence and frequency of which depended, at least in part, on extracellular Ca^{2+}.

The purpose of the present study was to characterize the variations of $[Ca^{2+}]_i$ and the occurrence of oscillations in rat freshly isolated tracheal smooth muscle cells in response to muscarinic stimulation. In order to identify the source of calcium involved in $[Ca^{2+}]_i$ oscillations, the effects of extracellular removal, of verapamil, a voltage-dependent Ca^{2+} channel blocker, and of thapsigargin, a sarcoplasmic Ca^{2+}-ATPase blocker, on the $[Ca^{2+}]_i$ response were investigated. In addition, since various subtypes of muscarinic receptor have been identified in the lung (Barnes, 1993), we determined the cholinoceptor subtypes involved in the muscarinic response by testing the inhibitory effect of the competitive antagonists on both the rise in $[Ca^{2+}]_i$ in isolated cells and isometric contraction of tracheal rings.

Methods

Tissue preparation

Rat tracheae were obtained from male Wistar rats 10–15 weeks old, weighing 300 to 400 g. For each experiment, a rat was anaesthetized by intraperitoneal administration of 400 mg ethylcarbamate. Heart and lungs were removed *en-bloc*, and the trachea was rapidly removed. For isometric contraction measurements, the trachea was cut into 4 rings of similar 3 mm diameter and 3–4 mm in length as previously described (Ben-Jebria *et al*., 1993). For fluorescence measurements of $[Ca^{2+}]_i$ in freshly isolated cells, the muscular strip located on the dorsal face of the trachea was further dissected under binocular control. The epithelium was removed and the epithelium-free muscular strip was cut in several pieces (1×1 mm) and incubated for 10 min in low-Ca^{2+} (200 μM) physiological saline solution (PSS, composition given below). Tissue was then incubated in low-Ca^{2+} PSS containing 1.0 mg ml^{-1} collagenase, 0.7 mg ml^{-1} pronase, 0.06 mg ml^{-1} elastase and 3 mg ml^{-1} bovine serum albumin at 37°C for two successive periods of 25 min. After this time, the solution was removed and the tracheal muscle pieces were incubated again in a fresh enzyme-free solution and triturated with a fire polished Pasteur pipette to release cells. Cells were stored attached to glass cover slips at 4°C in PSS containing 0.8 mM Ca^{2+} and used on the same day.

Fluorescence measurement and estimation of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were monitored fluorometrically by use of the Ca^{2+}-sensitive probe indo-1 as described previously (Guibert *et al*., 1996). Freshly isolated cells were loaded with indo-1 by incubation in PSS containing 1 μM indo-1 penta-acetoxymethyl ester (indo-1 AM) for 25 min at room temperature and then washed in PSS for 25 min. Coverslips with attached cells were then mounted in a perfusion chamber and continuously superfused at room temperature. The recording system included a Nikon Diaphot inverted microscope fitted with epifluorescence (Nikon France, Charenton-le-pont, France). A single cell was illuminated at 360 ± 10 nm. Emitted light from a window slightly larger than the cells was counted simultaneously at 405 nm and 480 nm by two photomultipliers (P100, Nikon). Voltage signals at each wavelength were stored in an IBM-PC computer for subsequent analysis. The fluorescence ratio (405/480) was calculated on-line and displayed with the two voltage signals on a monitor. $[Ca^{2+}]_i$ was estimated from the 405/480 ratio (Grynkiewicz *et al*., 1985) by use of a calibration for indo-1 determined within cells (Guibert *et al*., 1996).

Physiological saline solution (PSS) contained (in mM): NaCl 130, KCl 5.6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11 and HEPES 10; pH 7.4 with NaOH. Ca^{2+}-free PSS was prepared by replacing $CaCl_2$ by 0.4 mM ethyleneglycol-bis[β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA). Acetylcholine (ACh) or carbachol were applied to the tested cell by a 30 s pressure ejection from a glass pipette located close to the cell. No changes in $[Ca^{2+}]_i$ were observed during test ejections of PSS (data not shown). Generally, each record of $[Ca^{2+}]_i$ response to acetylcholine or carbachol alone or in the presence of an additional substance was obtained from a different cell. Each type of experiment was repeated for the number of cells indicated in the text.

Isometric contraction measurement

Isometric contraction was measured in airway smooth muscle rings that were mounted between two stainless steel clips in vertical 20 ml organ baths of an isolated organ bath system attached to a computer (IOS_1, EMKA Technologies, Paris, France) as described previously (Ben-Jebria *et al*., 1993). Baths were filled with Krebs-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and D-glucose 11.1; pH 7.4) maintained at 37°C and bubbled with a 95% O_2-5% CO_2 gas mixture. The upper stainless clip was connected to an isometric force transducer (EMKA Technologies). Tissues were set at optimal length by equilibration against a passive load of 1.5 g, as determined previously for this type of preparation (Ben-Jebria *et al*., 1993). At the beginning of each experiment, a supramaximal stimulation with acetylcholine (ACh, 10^{-3} M final concentration in the bath) was administered to each of the rings to elicit a reference response that was used to normalize subsequent contractile responses. After the rings had been washed with fresh Krebs-Henseleit solution to eliminate the ACh response, a cumulative concentration-response curve (CCRC) to carbachol was constructed. Carbachol was used as a convenient cholinoceptor agonist since, unlike ACh, it is not metabolized by acetylcholinesterase and thus can be used to construct steady-state cumulative concentration-response curves. Fifteen minutes before the beginning of the CCRC,

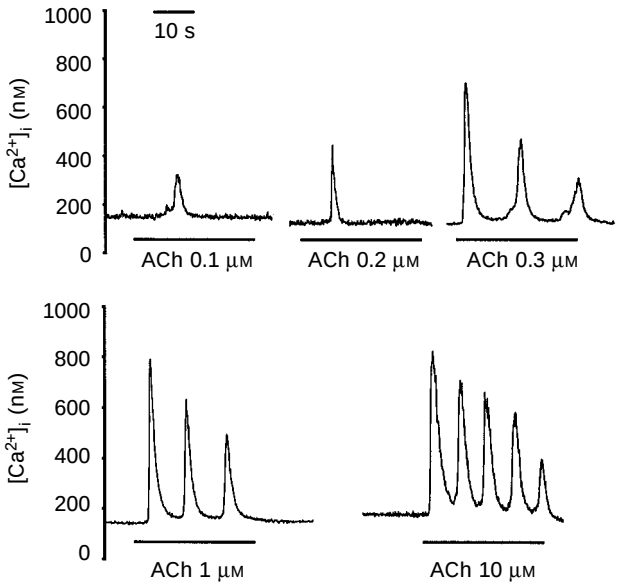

Figure 1 Effect of ACh on the intracellular concentration ($[Ca^{2+}]_i$) in rat freshly isolated tracheal smooth muscle cells loaded with indo-1. Ejection of ACh (30 s, concentration indicated on the trace) induced an initial graded $[Ca^{2+}]_i$ peak followed, for concentrations higher than ~0.2 μM, by oscillations of decreasing amplitude superimposed on a plateau phase. Each trace is representative of 24 to 32 different cells.

the desired muscarinic antagonist was administered at 3 different concentrations to 3 of the 4 rings in order for pA_2 values to be calculated. The unexposed ring served as temporal control.

Chemicals and drugs

Collagenase (type CLS1) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Pronase (type E), elastase (type 3), bovine serum albumin, acetylcholine (ACh), carbachol, atropine, thapsigargin (TG), verapamil and caffeine were purchased from Sigma (Saint Quentin Fallavier, France). Indo-1 AM was from Calbiochem (France Biochem, Meudon, France). Methoctramine, 4-diphenyl acetoxy N-methyl piperidine (4-DAMP) and pirenzepine (PZ) were obtained from Research Biochemical International (Natick, MA, U.S.A.).

Indo-1 AM, and thapsigargin were dissolved in dimethyl sulphoxide (DMSO). The maximal concentration of DMSO used in our experiments was $<0.1\%$ and had no effect on the resting value of the $[Ca^{2+}]_i$ nor on the variation of the $[Ca^{2+}]_i$ induced by ACh (data not shown).

Statistical and data analysis

Results of $[Ca^{2+}]_i$ are expressed as the mean $\pm$ s.e.mean with n the sample size. Significance was tested by means of Student's t test. When multiple comparisons were done, an analysis of variance (one-way ANOVA) was first performed and, when significant, Student's modified t tests were carried out by use of the Bonferroni method. When concentration-response curves were constructed, the mean $[Ca^{2+}]_i$ values calculated on n cells for the various concentrations of agonist were fitted by a sigmoidal Boltzman's equation and a mean EC_{50} was derived. To determine the concentration of antagonist at which the maximal increase in $[Ca^{2+}]_i$ was reduced by 50%, i.e., the IC_{50}, the amplitude of the peak of $[Ca^{2+}]_i$, expressed as the percentage of $[Ca^{2+}]_i$ peak amplitude of control cells, was plotted against the agonist concentration and fitted again by a sigmoidal Boltzman's equation. Competitive antagonism in contraction experiments was assessed according to the method of Arunlakshana & Schild (1959). In each experiment, one dose-ratio value (DR) was obtained for each concentration of antagonist. Two to 3 experiments were performed for each

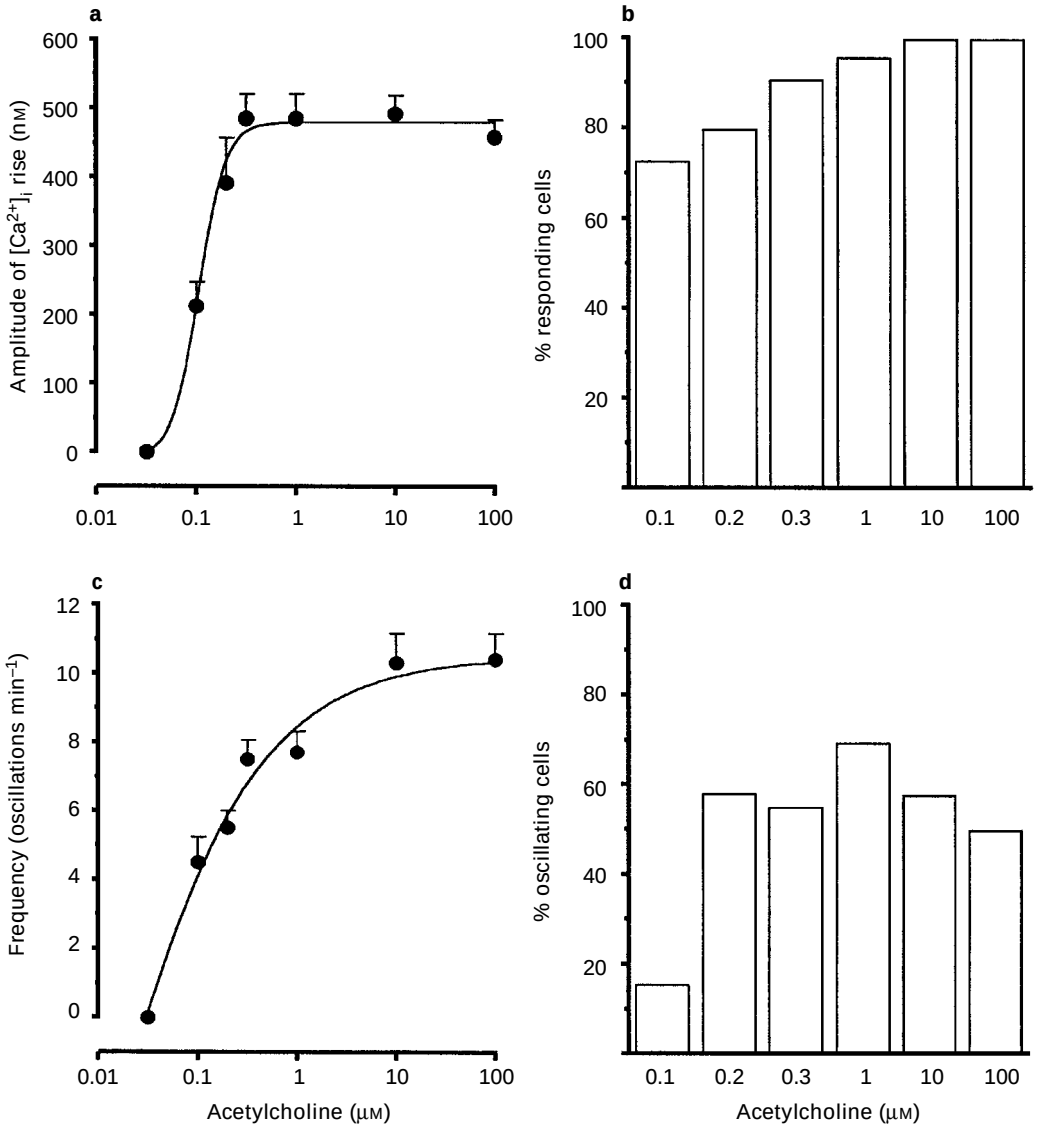

Figure 2 Concentration-response characteristics of the ACh-induced $[Ca^{2+}]_i$ response in rat freshly isolated tracheal smooth muscle cells loaded with indo-1. (a) The net amplitude of $[Ca^{2+}]_i$ rise (the maximal increase above resting baseline concentration) was plotted versus ACh concentration (abscissa scale). Each symbol represents the mean value of $[Ca^{2+}]_i$ rise calculated from 15 to 34 cells; vertical lines indicate s.e.mean. (b) Relationship between ACh concentration (abscissa scale) and the percentage of responding cells (ordinate scale). (c) Relationship between ACh concentration (abscissa scale) and the frequency of oscillations min^{-1} calculated in 10 to 15 cells generating oscillations in $[Ca^{2+}]_i$; vertical lines indicate s.e.mean. (d) Relationship between ACh concentration (abscissa scale) and the percentage of cells generating oscillations in $[Ca^{2+}]_i$ (% oscillating cells, ordinate scale).

antagonist. pA_2 values were denoted as the X-intercept of the Schild regression, according to $pA_2 = -\log$ {[antagonist]/(DR − 1)}. The slope and the correlation of the Schild regression were calculated. It was verified that the slope was not statistically different from unity. Results were considered significant at $P < 0.05$.

Results

Effect of muscarinic agonists on [Ca^{2+}]$_i$ in isolated tracheal smooth muscle cells

In rat tracheal smooth muscle cells, the mean resting concentration of [Ca^{2+}]$_i$ was 135 ± 2.6 nM ($n = 145$). Stimulation by ACh caused a rapid and graded increase in [Ca^{2+}]$_i$ which gradually declined (Figure 1). This first peak was followed, when the ACh concentration increased, in approximately half of the cells, by successive peaks of decreasing amplitude ([Ca^{2+}]$_i$ oscillations) (Figure 1) or by a plateau phase which remained above baseline for as long as the muscarinic stimulation persisted. The amplitude of the first [Ca^{2+}]$_i$ peak, the occurrence as well as the frequency of the [Ca^{2+}]$_i$ oscillations depended on the ACh concentration (Figure 1). Between 0.03 and 10 μM, the amplitude of the peak (i.e., the maximal increase in [Ca^{2+}]$_i$ above baseline observed during the transient phase), as well as the percentage of responding cells gradually increased with ACh concentration (Figure 2a,b). The ACh concentration that induced half maximal [Ca^{2+}]$_i$ rise in responding cells, i.e. the EC_{50}, was 0.13 μM. In contrast, the occurrence of [Ca^{2+}]$_i$ oscillations was an all-or-none phenomenon. For ACh concentrations below 0.2 μM, the percentage of cells exhibiting [Ca^{2+}]$_i$ oscillations was below 20% and increased sharply to approximately 50–60% and remained consistent for higher ACh concentrations (Figure 2d). Finally, for an ACh stimulation of a constant duration, the frequency of [Ca^{2+}]$_i$ oscillations increased with concentration, up to 10 min^{-1} for 100 μM ACh (Figure 2c). When the tracheal isolated myocytes were stimulated by carbachol, the amplitude of the [Ca^{2+}]$_i$ response as well as the concentration response relationship were similar to those observed with ACh (data not shown).

Effect of external calcium removal and of verapamil on [Ca^{2+}]$_i$ responses to cholinoceptor agonists

Superfusion of the cells with Ca^{2+}-free PSS containing 0.4 mM EGTA reduced only slightly the resting [Ca^{2+}]$_i$ concentration from 120 ± 5.7 nM ($n = 15$) to 109 ± 4.7 nM ($n = 17$) ($P > 0.05$, NS). Whatever the cholinoceptor agonist, carbachol or ACh, at a concentration of 10 μM, the amplitude of the peak increase in [Ca^{2+}]$_i$ value was not significantly different in the presence or in the absence of external Ca^{2+} (417 ± 26, $n = 15$ and 403 ± 32 nM, $n = 17$, respectively in response to carbachol; 458 ± 25, $n = 34$ and 403 ± 32 nM, $n = 17$, respectively in response to ACh, Figure 3b). Removal of extracellular Ca^{2+} did not abolish the oscillations (Figure 3a). When the sustained [Ca^{2+}]$_i$ response was estimated as the averaged amplitude of the plateau phase onto which oscillations were superimposed, again, there was no significant difference in the presence or absence of external Ca^{2+} (46 ± 5.9 and 39 ± 5.1 nM, respectively in response to carbachol; 63 ± 5.8 and 57 ± 11 nM, respectively in response to ACh, Figure 3b). Verapamil, 10 μM did not modify either the mean value of the peak, or that of the plateau and oscillations of the [Ca^{2+}]$_i$ response to ACh 10 μM compared to control (Figure 3a). The peak increase in [Ca^{2+}]$_i$ was 655 ± 92 nM, $n = 9$ and 605 ± 88, $n = 8$ (NS), and the amplitude of the plateau phase was 87 ± 12, and 75 ± 13 nM (NS), in the presence and absence of verapamil, respectively. A similar result was obtained when cells were stimulated with carbachol 10 μM ($n = 7$, not shown). In control experiments, it was verified that 10 μM verapamil did block the [Ca^{2+}]$_i$ increase induced by 110 mM KCl ($n = 13$, data not shown).

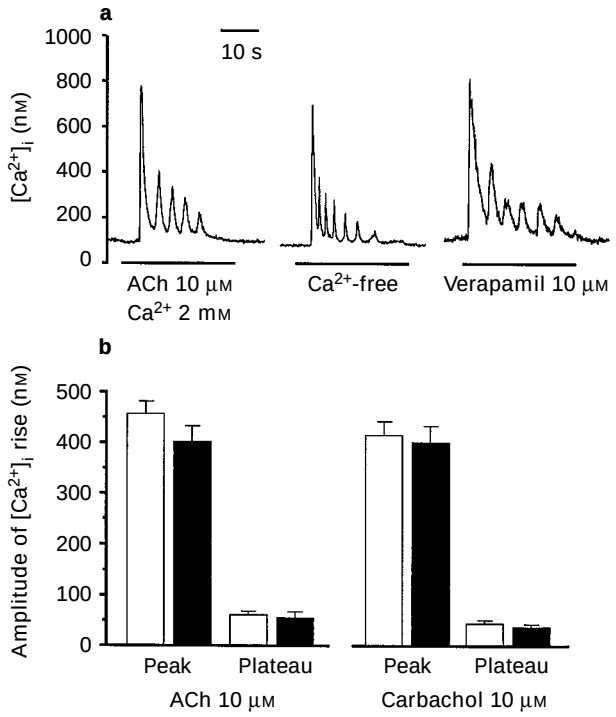

Figure 3 Effect of external calcium removal and of verapamil on [Ca^{2+}]$_i$ response in rat freshly isolated tracheal smooth muscle cells loaded with indo-1. (a) Typical responses to ACh (10 μM) in control conditions, in Ca^{2+}-free solution (0.4 mM EGTA for 10 min) and in the presence of verapamil (10 μM for 10 min); ACh-induced [Ca^{2+}]$_i$ oscillations were recorded in all conditions. (b) Both ACh- and carbachol-induced an initial rise in [Ca^{2+}]$_i$ (peak) and sustained rise above baseline (plateau) were not different in the presence (open columns) of external Ca^{2+} (2 nM) or in its absence (solid columns, with 0.4 mM EGTA). Each column represents a mean value calculated from 15 to 34 cells; vertical lines indicate s.e.mean.

Effect of thapsigargin on [Ca^{2+}]$_i$ responses to ACh and caffeine

In these experiments, cells were pre-incubated in PSS with thapsigargin. Both the first [Ca^{2+}]$_i$ peak and [Ca^{2+}]$_i$ oscillations in response to 10^{-5} M ACh progressively declined and were completely abolished after an incubation of 12–15 min with thapsigargin (1 μM) in Ca^{2+}-free PSS with 0.4 mM EGTA ($n = 10$) (Figure 4a).

In a subsequent series of experiments, we studied the effect of caffeine, an agent that releases intracellular Ca^{2+} ions in airway smooth muscle. Caffeine (0.1–5 mM) evoked only one transient rise in [Ca^{2+}]$_i$ of increasing amplitude from 342 ± 106 nM ($n = 9$) to 712 ± 58 nM ($n = 19$). As for ACh, thapsigargin (1 μM) in Ca^{2+}-free PSS with 0.4 mM EGTA did block the caffeine-induced transient rise in [Ca^{2+}]$_i$ within 12–15 min (Figure 4b). However, the pattern of the [Ca^{2+}]$_i$ response to caffeine in airway smooth muscle was completely different from that to ACh since, in our experimental conditions, caffeine did not produce oscillations (Figure 4b).

Effects of muscarinic antagonists on [Ca^{2+}]$_i$ responses to ACh in isolated tracheal smooth muscle cells

The effect of atropine, pirenzepine, methoctramine and 4-DAMP was tested on the [Ca^{2+}]$_i$ response to 10 μM ACh. The inhibitory effect was assessed by comparing the response in the presence and absence of the appropriate antagonist. The rise in [Ca^{2+}]$_i$ induced by ACh in the presence of each concentration of the desired antagonist was expressed as a percentage of that

obtained in the absence of any antagonist (control). Inhibition curves for the four muscarinic antagonists are summarized in Figure 5. IC_{50} values for atropine, 4-DAMP and pirenzepine were 13 nM, 20 nM and 2 μM, respectively. Methoctramine up to the concentration of 10 μM caused only a 40% inhibition.

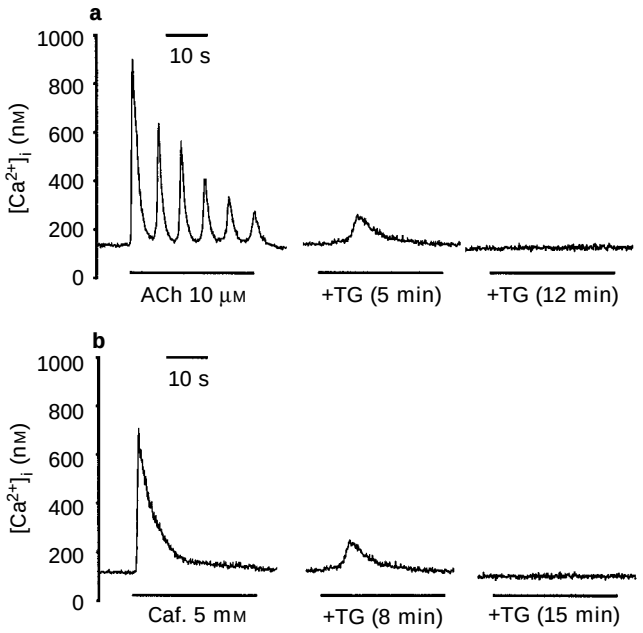

Figure 4 Effect of thapsigargin (TG) on the $[Ca^{2+}]_i$ response induced by ACh and caffeine (Caf) in the absence of external Ca^{2+} ions in rat freshly isolated tracheal smooth muscle cells loaded with indo-1. (a) Superfusion of tracheal myocytes with TG (1 μM) time-dependently inhibited $[Ca^{2+}]_i$ oscillations evoked by ACh (10 μM) and abolished the response within 12–15 min. (b) Ejection of Caf (5 mM) for 30 s induced only one transient rise in $[Ca^{2+}]_i$ and superfusion of tracheal myocytes with TG (1 μM) also time-dependently inhibited this transient $[Ca^{2+}]_i$ rise. In each panel, the first record is the control response obtained in the absence of TG. The duration of exposure to TG before ACh or Caf ejection is shown in parentheses. Each trace was recorded from a different cell and is typical of 10 to 19 cells.

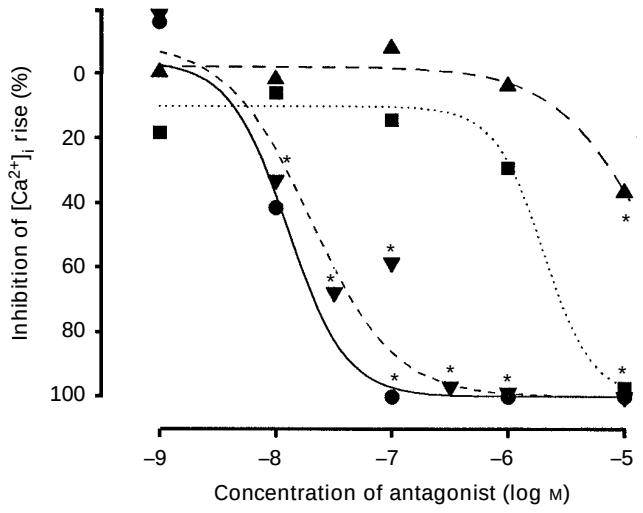

Figure 5 Inhibition curves of ACh (10 μM)-evoked $[Ca^{2+}]_i$ rise for 4 muscarinic cholinoceptor antagonists in rat freshly isolated tracheal smooth muscle cells loaded with indo-1. Inhibition (ordinate scale) is the percentage of the net amplitude of $[Ca^{2+}]_i$ rise in the presence of the antagonist versus that in the absence of the antagonist. Abscissa scale: concentration (M) of antagonist on a log scale. Each symbol represents a mean value calculated from 11–19 cells. For sake of clarity, vertical lines (s.e.mean) are not indicated. (●) Atropine; (▼) 4-DAMP; (■) pirenzepine and (▲) methoctramine. $*P<0.05$ between the response in the presence and that in the absence (control) of the antagonist.

Effects of muscarinic antagonists on contractile response of tracheal rings to carbachol

In these experiments, the effect of muscarinic antagonists on CCRC for carbachol was assessed at the following concentrations. Atropine and 4-DAMP were tested at 3, 10 and 30 nM; pirenzepine was used at 0.3, 1 and 3 μM, and methoctramine at 1, 3 and 10 μM. For these concentrations, all of the antagonists produced a rightward shift of the carbachol CCRC (Figure 6) and pA_2 values are presented in Table 1.

Discussion

The present study indicates that muscarinic stimulation of rat isolated tracheal smooth muscle cells induce $[Ca^{2+}]_i$ oscillations. The occurrence of these oscillations depends on the graded amplitude of the first $[Ca^{2+}]_i$ rise and their frequency may play a role in the amplitude of the mechanical activity in response to cholinergic stimulation. Both the $[Ca^{2+}]_i$ and the contractile responses are primarily dependent on activation of the M_3 receptor subtype.

The first component of the muscarinic-induced $[Ca^{2+}]_i$ signal was a transient and rapid rise in $[Ca^{2+}]_i$ the amplitude of which depended on the concentration of the muscarinic agonist. Such a graded $[Ca^{2+}]_i$ response to muscarinic cholinoceptor stimulation has already been observed in both cultured airway smooth muscle cells (Yang *et al*., 1991; 1993b) and freshly isolated cells from the porcine (Liu & Farley, 1996a) or bovine (Kajita & Yamagushi, 1993) trachealis. As in previous studies in which the effects of various agonists on the $[Ca^{2+}]_i$ response were examined in freshly or cultured airway smooth muscle cells (Murray & Kotlikoff, 1991; Yang *et al*., 1991; 1993a,b; Kajita & Yamagushi, 1993; Marsh & Hill, 1993; Amrani *et al*., 1995; Tolloczko *et al*., 1995), we found the first peak to be dependent on intracellular calcium release, since it was not altered in the absence of external calcium or in the presence of the voltage-dependent Ca^{2+}-channel blocker verapamil but was blocked by the endoplasmic Ca^{2+}-ATPase blocker thapsigargin. Since IP_3 is the Ca^{2+}-releasing second messenger coupled to muscarinic stimulation (Roffel *et al*., 1990; Yang *et al*., 1991; Al-Hassani *et al*., 1993), this result suggests that, in airway as in other smooth muscles such as the portal vein (Loirand *et al*., 1994), Ca^{2+} is released from intracellular stores in a quantal manner with an increasing fraction of the store becoming involved in the overall $[Ca^{2+}]_i$ signal as the concentration of ACh and hence of IP_3 increases. Moreover, the maximal amplitude of the value of the first increase in $[Ca^{2+}]_i$ induced by ACh in isolated myocytes was close to that required to activate fully the contractile apparatus in chemically skinned fibres of airway smooth muscle (Savineau & Marthan, 1994).

The second component of the muscarinic-induced $[Ca^{2+}]_i$ signal was, in approximately 50 to 60% of the cells, successive peaks of decreasing amplitude ($[Ca^{2+}]_i$ oscillations) superimposed on a plateau phase. It is likely that $[Ca^{2+}]_i$ oscillations are specific cytosolic oscillators since, as for the first $[Ca^{2+}]_i$ peak, they were not altered in the absence of external calcium or in the presence of the voltage-dependent Ca^{2+}-channel blocker verapamil. These oscillations seem to be related to a repetitive Ca^{2+} release from intracellular Ca^{2+} stores since they disappeared following treatment with thapsigargin, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase in smooth muscle which prevents the refilling of these stores (Gonzalez De La Fuente *et al*., 1995; Amrani *et al*., 1995). Moreover, these oscillations appear to involve a specific type of Ca^{2+} release channel in the sarcoplasmic reticulum of the airway smooth muscle cell, i.e. the IP_3 receptor-channel, for the following reasons. Firstly, caffeine, which also releases Ca^{2+} from intracellular stores in airway smooth muscle but via ac-

tivation of the ryanodine-sensitive Ca^{2+} release channel (Chopra *et al*., 1991), failed to produce $[Ca^{2+}]_i$ oscillations. Secondly, the occurrence of $[Ca^{2+}]_i$ oscillations was an all-or-none phenomenon. For ACh concentrations below 0.2 μM i.e. for $[Ca^{2+}]_i$ values below ~300 nM (Figure 2a), the percentage of cells exhibiting $[Ca^{2+}]_i$ oscillations was low and increased sharply and remained consistent for higher ACh and hence $[Ca^{2+}]_i$ concentrations (Figure 2d). This phenomenon is in agreement with the complex positive and negative feedback control of the IP_3 receptor-channel by Ca^{2+} (Iino, 1990). The amplitude of each $[Ca^{2+}]_i$ spike may represent the balance between Ca^{2+} release, the loss of Ca^{2+} from the cell and the sequestration of Ca^{2+} into internal stores. The termination of the spike would occur when the release process is inactivated, allowing the extrusion of Ca^{2+} from the cell and the sequestration of Ca^{2+} into intracellular stores. The decrease in the amplitude of $[Ca^{2+}]_i$ oscillations probably results from a gradual reduction in the Ca^{2+} store content due to the loss of Ca^{2+}. The reason why $[Ca^{2+}]_i$ oscillations were observed only in 50–60% of cells is not clear. This percentage did not increase when the concentration of ACh increased, suggesting that it represented the maximal percentage of cells exhibiting $[Ca^{2+}]_i$ oscillations. This may reflect the heterogeneity of myocytes located in the smooth muscle of the rat trachea.

This second component of the muscarinic-induced $[Ca^{2+}]_i$ signal and in particular the occurrence of $[Ca^{2+}]_i$ oscillations that are largely independent of the calcium source is quite different from previous findings in cultured airway myocytes (Murray & Kotlikoff, 1991; Yang *et al*., 1991; 1993a,b; Marsh & Hill, 1993; Amrani *et al*., 1995; Tolloczko *et al*., 1995). In all of these latter studies, the first $[Ca^{2+}]_i$ peak was followed by a steady state plateau which depended on extracellular Ca^{2+}. This difference may reflect changes in the control of the IP_3 receptor-channel induced by the cell culture. This hypothesis would be supported by the fact that agonist-induced $[Ca^{2+}]_i$ oscillations were observed in single smooth muscle cells that, in general, were freshly isolated (Guibert *et al*., 1996; Kohda *et al*., 1996; Liu & Farley, 1996a,b). However, our results are also different from those of Kajita & Yamagushi (1993) who observed, in bovine freshly isolated tracheal smooth muscle cells, that the sustained phase following carbachol stimulation was a

Table 1 Inhibition of carbachol-induced contraction of rat trachea by cholinoceptor antagonists

Antagonist	pA_2	*Slope*	*Correlation* (r)
Atropine	9.20	1.24 (0.23)	0.90
Pirenzepine	6.69	1.06 (0.19)	0.94
4-DAMP	8.75	1.29 (0.15)	0.95
Methoctramine	6.27	1.10 (0.07)	0.99

pA_2 are calculated by use of Schild plots. Values in parentheses show s.d.

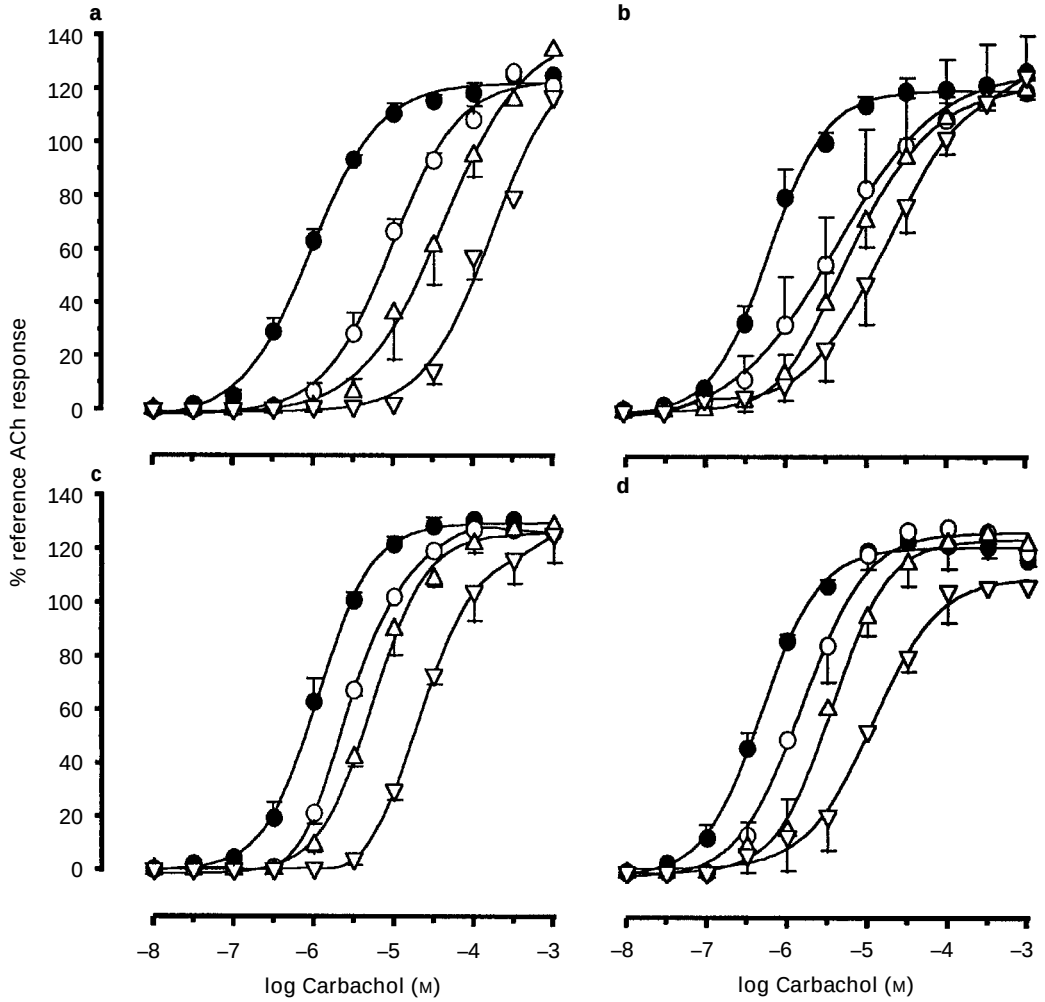

Figure 6 Mean cumulative concentration-response curves for carbachol in the absence (●) and in the presence (open symbols) of muscarinic cholinoceptor antagonists at various concentrations in rat isolated tracheal rings. Ordinate scale: contractile response to each concentration of carbachol expressed as a percentage of a reference response to ACh (1 mM) administered to each of the rings at the beginning of the experiment. (a) Atropine (○) 3 nM; (△) 10 nM; (▽) 30 nM. (b) 4-DAMP (○) 3 nM; (△) 10 nM; (▽) 30 nM; (c) pirenzepine (○) 300 nM; (△) 1 μM; (▽) 3 μM; (d) methoctramine (○) 1 μM; (△) 3 μM; (▽) 10 μM. Vertical lines indicate s.e.mean.

steady state plateau that depended on extracellular calcium and was suppressed by D600 (methoxyverapamil), a voltage-dependent Ca^{2+}-channel blocker. This discrepancy may be due to a species difference, or alternatively may be explained by variations in the duration of muscarinic stimulation. Indeed, these authors observed the effect of the Ca^{2+}-free medium and of D600 during prolonged stimulation of fura 2-loaded cells whereas, in our experiments, indo 1-loaded cells were stimulated for 30 s.

In agreement with our results, ACh-induced $[Ca^{2+}]_i$ oscillations have been recently described in porcine freshly isolated tracheal smooth muscle cells, with the Ca^{2+}-dependent chloride current measured in patch clamp experiments as an index of cytosolic Ca^{2+} concentration changes (Liu & Farley, 1996a,b). The involvement of IP_3 in the oscillations, suggested in the present study, was directly demonstrated since IP_3 applied intracellularly in the patch pipette mimicked ACh-induced oscillations in the Ca^{2+}-dependent chloride current. As in the present study, the frequency of Ca^{2+}-dependent chloride current oscillations increased with the ACh concentration, suggesting that oscillations may represent a digitalisation of the calcium signal allowing a frequency-dependent control of the cellular response (Berridge, 1993). However, whereas in swine tracheal smooth muscle cells, maintenance of the oscillations required Ca^{2+} influx in part through voltage-operated channel (Liu & Farley, 1996a), Ca^{2+} influx did not play a direct role in the $[Ca^{2+}]_i$ response to muscarinic stimulation in our experiments. Our findings are consistent with the observation that contraction of tracheal smooth muscle is little dependent on extracellular calcium (Farley & Miles, 1978) and that extracellular Ca^{2+} influx through voltage-operated channels plays a minor role in contractions induced by muscarinic agonists (Al-Hassani *et al*., 1993). The discrepancy in terms of calcium-dependence between our results and those of Liu & Farley (1996a) may again be due to species differences, or alternatively may be explained by variations in the duration of muscarinic stimulation. Although Ca^{2+} influx did not play a role in the $[Ca^{2+}]_i$ response to muscarinic stimulation in our experiments, it may play an indirect one in refilling the intracellular calcium stores, as it has been suggested in dog trachea (Bourreau *et al*., 1991).

To assess the role of these $[Ca^{2+}]_i$ oscillations in the mechanical activity further, we have determined the cholinoceptor subtypes involved in the muscarinic response by testing the inhibitory effect of competitive antagonists on both $[Ca^{2+}]_i$ rise in isolated cells and isometric contraction of tracheal rings. Various subtypes of muscarinic receptor have been identified in the lung (Barnes, 1993; Haddad *et al*., 1994) and M_2 and M_3 receptor subtypes have been found in rat airway smooth muscle (Gies *et al*., 1989), although there are large interspecies variations in subtype expression. Functional studies have shown a major role of the M_3 receptor subtype in bronchoconstriction (Post *et al*., 1991; Garsen *et al*., 1993) and, although radioligand binding studies have indicated a high proportion of M_2 receptors in airway smooth muscle (Fryer & El-Fakahany, 1990; Haddad *et al*., 1994), the role of this subtype remains rather unclear (Fernandes *et al*., 1992; Eglen *et al*., 1994; Roffel *et al*., 1995). We assessed the action of four muscarinic cholinoceptor antagonists over a wide range of concentrations on the $[Ca^{2+}]_i$ response to ACh. A comparison of the IC_{50} values of each antagonist shows that 4-DAMP, a selective M_3 antagonist, was much more potent than the M_1 antagonist, pirenzepine and the M_2 antagonist, methoctramine. The pA_2 values calculated, in contraction experiments, for each of the antagonists are in good agreement with previously published data in rat and mouse trachea (Post *et al*., 1991; Garssen *et al*., 1993). Furthermore, since the Schild regressions were linear and since the slope was not different from unity for each of the antagonists, the pA_2 value can be considered as an estimate of the pK_B (Kenakin, 1993) and thus can be compared to its value determined in binding studies for the different muscarinic receptor subtypes, M_1, M_2 and M_3 (Eglen *et al*., 1994). The pA_2 value for 4-DAMP was close to its pK_B for the M_3 receptor subtype. In contrast, pA_2 values for pirenzepine and methoctramine did not correlate well with their pK_B determined for M_1 and M_2 receptor subtypes, respectively, but were rather close to their pK_B for the M_3 receptor subtype. This confirms that the muscarinic-induced contraction involves M_3 receptors. These results also indicate that atropine and 4-DAMP have a high affinity for the airway smooth muscle cholinoceptor involved in the contractile response, whereas pirenzepine and methoctramine have a low affinity. This difference in the affinity of the various antagonists for the receptor subtype involved in the contraction parallels that observed for the $[Ca^{2+}]_i$ response. The 2 log shift of pA_2 value between atropine or 4-DAMP and pirenzepine or methoctramine is similar to that observed for the $[Ca^{2+}]_i$ inhibition curves (Figure 5), further confirming the involvement of the M_3 receptor subtype in the $[Ca^{2+}]_i$ response.

In conclusion, muscarinic stimulation of rat isolated tracheal smooth muscle cells induces $[Ca^{2+}]_i$ oscillations depending on the graded amplitude of the first $[Ca^{2+}]_i$ rise. The frequency of these oscillations is concentration-dependent. Both the $[Ca^{2+}]_i$ and the contractile responses are primarily dependent on activation of the M_3 receptor subtype.

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