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Pharmacological and Molecular Characterization of Muscarinic Receptors in Cat Esophageal Smooth Muscle[1]

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ABSTRACT

The muscarinic receptor subtypes that mediate cholinergic responses in cat esophageal smooth muscle were examined. Antagonist effects on carbachol-induced and nerve-evoked contractions were studied *in vitro* using muscle strips from the distal esophagus. Antagonists displayed similar relative selectivities in suppressing carbachol and nerve-mediated responses as follows: 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) > zamifenacin > *para*-fluoro-hexahydrosiladiphenidol > pirenzepine > AF-DX 116 > methoctramine, indicating that these responses are mediated by the same receptor subtype. 4-DAMP, pirenzepine and methoctramine effects on carbachol responses gave pA_2 values characteristic of the M_3 receptor in both the circular muscle (9.25 ± 0.12, 6.79 ± 0.09 and 6.04 ± 0.11, respectively) and longitudinal muscle (9.46 ± 0.14, 7.25 ± 0.07 and 6.10 ± 0.06, respectively). Reverse transcription-polymerase chain reaction analysis was done using primer sequences based on the cloned human muscarinic receptor subtypes. Messenger RNA for the m_3 receptor was readily identified, whereas m_2 was not detected in esophageal muscle, but was present in cardiac muscle. Sequence homology between the amplified products from cat tissue and the corresponding human m_2 and m_3 receptors genes were 93% and 89%, respectively. In the cat esophagus, the M_3 receptor mediates functional responses and messenger RNA for the corresponding molecular form of this receptor is abundant in this tissue.

Muscarinic receptors mediate cholinergic excitation in the distal smooth muscle esophagus. The overall contribution of this excitatory mechanism to normal esophageal peristalsis differs between species. Thus, atropine potently blocks swallow-induced peristalsis in the cat, the monkey and the human esophagus, but in the opossum, it has a more modest effect (Goyal and Paterson, 1989; Diamant, 1989). These findings are supported by *in vitro* studies in which nerve-mediated responses evoked by EFS of muscle strips from the cat or the human esophagus also are inhibited by atropine (Behar *et al*., 1989; Preiksaitis *et al*., 1994), whereas in the opossum, a significant atropine-resistant, noncholinergic, nonadrenergic contraction is seen (Crist *et al*., 1984).

Five subtypes of the muscarinic receptor (m_1–m_5) have been identified by molecular cloning techniques (Bonner *et al*., 1987; Peralta *et al*., 1987; Dorje *et al*., 1991). To date muscarinic receptor agonists or antagonist with sufficient selectivity to distinguish any one subtype from all the others have not been developed. However, four subtypes of the receptor (M_1–M_4) can be differentiated pharmacologically based on the pattern of selectivity for several muscarinic antagonists (Hulme *et al*., 1990; Caulfield, 1993; Eglen *et al*., 1996a; Dorje *et al*., 1991). Previous *in vivo* studies to characterize muscarinic receptor subtypes in the cat (Blank *et al*., 1989) and the opossum esophagus (Gilbert and Dodds, 1986) concluded that cholinergic activation accompanying peristalsis occurred mainly via a M_2-mediated mechanism. This conclusion was based in part on the lack of effect of pirenzepine, a M_1 receptor antagonist, *vs.* the greater selectivity of 4-DAMP, originally designated as a M_2-selective antagonist. 4-DAMP is now known to be more selective for the M_3 receptor (Eglen *et al*., 1996a). Hence, it is more likely that the M_3 receptor mediates peristalsis *in vivo* in the cat and the opossum (Goyal, 1989).

However, in a recent *in vitro* study on isolated smooth muscle cells from the circular layer of the cat esophagus, Sohn *et al*. (1993) demonstrated that methoctramine, a selective M_2 antagonist, was more effective in antagonizing acetylcholine-stimulated cell shortening than *p*-HHSiD, a selective M_3 antagonist, and concluded that the response was

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ABBREVIATIONS: AF-DX-116, 11-[[[2-diethylamino-0-methyl]-1-piperidinyl]acetyl]-5,11-dihydrol-6H-pyridol[2,3,-b][1,4]benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxy-*N*-methylpipiridine; EFS, electrical field stimulation; hm2 and hm3, human m_2 and m_3 receptor genes, respectively; cm2 and cm3, cat m_2 and m_3 receptor genes, respectively; mRNA, messenger RNA; L-NNA, N^G-nitro-L-arginine; *p*-HHSiD, *para*-fluoro-hexahydrosiladiphenidol; RT-PCR, reverse transcription-polymerase chain reaction.

therefore M_2-mediated. In other regions of the gastrointestinal tract, receptor binding studies have shown that smooth muscle expresses both M_2 and M_3 receptors; however, the contractile response in most gastrointestinal smooth muscles is mediated mainly by the M_3 subtype (Eglen *et al*., 1996a). This holds true in several species and preparations, including guinea pig terminal ileum (Eglen *et al*., 1992b; Barocelli *et al*., 1994; Michel and Whiting, 1990b; Michel and Whiting, 1988b; Giraldo *et al*., 1988), rat terminal ileum (Lazareno and Roberts, 1989), canine terminal ileum (Shi and Sarna, 1997) and human colon (Kerr *et al*., 1995; Gomez *et al*., 1992). Thus, the studies of Sohn *et al*. (1993), which show that the functional response of the circular muscle from the esophageal body of the cat is mediated by the M_2 receptor, represent a noteworthy exception for gastrointestinal smooth muscles. This observation holds significant clinical importance because it implies that anticholinergic agents that have sufficient selectivity for the M_2 receptor could be used to target esophageal motor disorders, whereas undesirable M_3-mediated systemic effects could be minimized.

In the present study, we address this controversy by characterizing the muscarinic receptor subtype(s) that mediates cholinergic responses in the cat esophagus using smooth muscle strips studied *in vitro*. We consider separately the longitudinal and circular layers since significant differences in the distribution of muscarinic receptors between muscle layers may exist (Preiksaitis *et al*., 1996). Additionally, we examine the relative effectiveness of selective receptor antagonists on cholinergic nerve-mediated responses.

Methods

Animals and tissue retrieval. The experimental protocol was in accordance with the ethical guidelines of the Canadian Council of Animal Care and approved by the University of Western Ontario Animal Care Committee. Thirty-six cats of either sex weighing between 3.1 and 6.2 kg were euthanized with a lethal dose of phenobarbitol (100 mg/kg intraperitoneally). The abdomen and chest were opened and the esophagus was excised *en bloc* from the aortic arch distally to include a 2- to 3-cm portion of the proximal stomach and was placed in room-temperature Kreb's solution equilibrated with 5% CO_2 and 95% O_2. The Kreb's solution had the following composition (in mM): sodium 143, potassium 5.0, calcium 2.5, magnesium 1.2, chloride 128, phosphate 2.2, bicarbonate 24.9, sulfate 1.2, and glucose 10. In some experiments, a small full thickness biopsy (~1 by 3 mm) taken from the mid-distal third of the esophageal body muscle was obtained immediately on opening the chest. The mucosa was removed and the muscle was frozen on dry ice and stored at −70°C for subsequent RNA extraction. Samples of terminal ileum were obtained similarly, immediately after opening the abdomen. An equivalent sized portion of cardiac tissue, which included all layers, was obtained from the ventricular muscle immediately on opening the chest and used for RNA extraction as detailed below.

Tissue bath studies. The esophagus was freed of surrounding fascia, opened lengthwise and pinned to its approximate *in situ* dimensions. After removal of the mucosa by sharp dissection, longitudinally and circularly oriented strips of approximately the same size (0.2 × 1.0 cm) were prepared, with the aid of a magnifying glass, from the same region of the esophageal body, 1 to 3 cm above the lower esophageal sphincter muscular ring. Care was taken to ensure that the long axis of each strip followed the direction of the muscle fibers. Strips were mounted vertically in 10-ml jacketed organ baths containing Kreb's solution held at 37°C and continuously bubbled with 5% CO_2 and 95% O_2. One end of each strip was fixed to an electrode holder, and the other end was fastened by a silk tie to a Grass FT03 isometric force transducer coupled to a Grass 79E chart recorder (Grass Instruments, Quincy, MA).

After 1-hr equilibration, each strip was gently stretched by 1 to 2 mm until the maximum tension response to 1 μM carbachol was obtained. In all experiments, the maximum amplitude of contraction was recorded. Concentration-response curves were produced by exposing strips to 10^{-8} to 10^{-3} M carbachol in a cumulative manner, with each incremental concentration being added when the response to the previous concentration stabilized. The cumulative concentration-response curve for carbachol was similar to that obtained with single concentrations of drug with complete washout of the effect between challenges. Because carbachol is potentially active at nicotinic and muscarinic receptors on parasympathetic ganglia, the effects of the nitric oxide synthase inhibitor L-NNA and hexamethonium were examined. To minimize any such effects, L-NNA (100 μM) and hexamethonium (10 μM) were present in experiments examining muscarinic antagonist on the carbachol response. Although the antagonists used in this study were maximally effective within minutes of application, muscle strips were exposed to muscarinic receptor antagonists for ≥30 min before being rechallenged with carbachol, to ensure adequate tissue penetration and equilibration. We confirmed that no further effect was produced with longer exposure (data not shown). Usually, one concentration of antagonist was tested in each muscle strip to determine the effects on complete carbachol concentration-response relations. In some cases no more than two increasing concentrations of antagonists were tested in sequence, yielding identical results to those obtained with single antagonist concentrations. In experiments examining suppression of the response to a single carbachol concentration (1 μM), increasing concentrations of antagonists were studied in each strip as follows. After establishing the control response to 1 μM carbachol, each muscle strip was exposed for 30 min to the lowest concentration of antagonist tested and then rechallenged with 1 μM carbachol. When the maximum response was obtained, the tissue was washed by repeated changes of the Kreb's solution until base-line tension recovered. A higher concentration of the same antagonist was returned to the bath, equilibrated for 30 min, and rechallenged with 1 μM carbachol. The cycle was repeated until the carbachol response was maximally suppressed.

To assess the effects of muscarinic receptor antagonists on nerve-mediated responses, EFS was delivered *via* two platinum wire ring electrodes separated by 1 cm that encircled the circular muscle strip. The electrodes were directly coupled to a two-channel Grass S22 stimulator (Grass Instruments, Quincy, MA) that supplied 0.5-msec square wave pulses in 3-sec trains at 10 Hz and 50 to 80 V (supramaximal) applied every 180 to 240 sec. Two types of nerve-mediated responses were studied: (1) typical off-type contractions, which occurred after a brief latency following the cessation of EFS, and (2) on-type contractions, which occurred during EFS were studied after nitric oxide synthase was inhibited by the addition of 100 μM L-NNA. Muscarinic receptor antagonists were added in a cumulative manner, with effects being determined when the amplitude of the EFS-induced contraction stabilized and three consecutive responses varied by <5%. The maximum amplitude of these three contractions was recorded and compared with the average of three responses before the addition of antagonist.

RT-PCR and molecular cloning of cat m_2 and m_3 receptors. Total RNA was isolated from cat esophagus using the method of Chomczynski and Sacchi (1987). RNA samples were run out on agarose gels to verify integrity. One microgram of total RNA from each sample was reverse transcribed for 90 min at 42°C using random hexamers and Superscript RNase H- (GIBCO BRL, Gaithersburg, MD). The cDNA was diluted 2.5 times, and 5 μl was used in each 50 μl PCR reaction. PCR reactions were carried out for 35 cycles with 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2 μM of m_2 or m_3 primers and 0.2 μl of *Taq* DNA polymerase (ID Labs Biotechnology) in the reaction mixture. The timing of each cycle was 0.5 min at 94°C, 0.5 min at 58°C and 1 min at 72°C, followed by a

final 7-min extension at 72°C. PCR products (13 μl) were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Primers were selected based on the known sequences for the human m_2 and human m_3 genes, and were purchased from GIBCO BRL. The respective upstream and downstream primers for m_2 were 5′-GGTCAGCAATGCCTCAGTTA-3′ and 5′-CTTGGTGCCAATTCTGATGC-3′, and for m_3 were 5′-TGATGATCGGTCTGGCTTGG-3′ and 5′-TGCTGCTGTGGTCTTGGTCC-3′. The predicted PCR product sizes were 676 base pairs for m_2 and 441 base pairs for m_3. PCR products were purified using PCRapid (ID Labs Biotechnology), subcloned into the pGEM-T Vector (Promega, Madison, WI), and transformed into JM109 competent cells (Promega). Plasmid DNA was purified using the RPM kit (BIO 101). Clones containing the PCR inserts were identified by restriction digest and sequenced by the Queen's University Core Facility for Protein/DNA Chemistry (Kingston, Ontario, Canada).

Data analysis and statistics. Carbachol responses and the effects of muscarinic antagonists were analyzed using the curve-fitting facilities of Prism V 2.0 (GraphPAD Software, San Diego, CA). Using this program, EC_{50} and IC_{50} values were obtained from the sigmoidal dose-response relationship generated from the experimental data by nonlinear regression. The method of Arunlakshana and Schild (1959) was used to determine pA_2 values. Straight lines were fitted by least-squares linear regression. The slope of a straight line was considered to be not different from unity if the 95% confidence interval for the slope included −1. Results are reported as mean ± S.E. The number of cats studied is indicated by n. Statistical comparisons were made with the Student's t test. $P < 0.05$ was considered significant.

Drugs and materials. Carbachol (carbamyl choline), atropine sulfate and L-NNA were obtained from Sigma Chemical (St. Louis, MO). Methoctramine, 4-DAMP, pirenzepine and *p*-HHSiD were obtained from Research Biochemicals (Natick, MA). AF-DX-116 (11-[[[2-diethylamino-0-methyl]-1-piperidinyl]acetyl]-5,11-dihydrol-6*H*-pyridol[2,3,-*b*][1,4]benzodiazepine-6-one) was generously provided by Boehringer Ingleheim, Germany. Zamifenacin was a gift from Pfizer Central Research, Sandwich, UK. All drugs were prepared as concentrated stock solutions and diluted into Kreb's buffer just before use such that drugs were added to the 10 ml tissue bath in a volume of 10 to 100 μl.

Results

Antagonist effects on carbachol-mediated responses in circular and longitudinal muscle. Carbachol caused a concentration-dependent increase in tension in both the longitudinal and circular muscles. The mean $-\log EC_{50}$ values for the circular muscle was 6.17 ± 0.06 and 6.88 ± 0.07 for longitudinal muscle indicating a slightly greater agonist potency in the longitudinal layer ($P < .001$, $n = 10$). Since carbachol may also act at nicotinic and muscarinic receptors on parasympathetic ganglia, the net effect of this drug could be influenced by additional activation of inhibitory or excitatory nerve pathways. Neither 100 μM L-NNA (which inhibits nitric oxide synthase, thus blocking inhibitory nerve effects) nor 10 μM hexamethonium, alone or in combination, had a significant effect on the maximum tension response or the EC_{50} for carbachol in longitudinal or circular muscle strips (table 1).

The effects of 6 muscarinic receptor antagonists on the tension response to 1 μM carbachol in circular and longitudinal muscle are shown in figure 1, A and B, respectively. The effectiveness of this group of antagonists was similar in circular and longitudinal muscle with 4-DAMP > zamifenacin > *p*-HHSiD > pirenzepine > AF-DX-116 > methoctramine. As can be seen in figure 1C and table 2, the IC_{50} values of these antagonists were similar in both muscle layers: the relationship of $-\log IC_{50}$ of the antagonists in each muscle layer is best described by a straight line with slope = 0.90 ± 0.05 ($r^2 = .98$), which is not significantly different from unity. A more detailed examination of the antagonism of the carbachol dose-response relationship in circular and longitudinal muscle strips was done using pirenzepine, methoctramine and 4-DAMP as prototypic, partially selective antagonists for M_1, M_2/M_4 and M_3/M_1 receptors, respectively. All three antagonists produced parallel rightward displacements of the dose-response curves (fig. 2), and Schild analysis yielded regression lines with slopes not significantly different from unity (fig. 3 and table 3). The resulting pA_2 values are provided in table 3 and demonstrate a high affinity of the receptor mediating carbachol contractions for 4-DAMP and a lower affinity for pirenzepine and methoctramine. In some preparations, methoctramine has been found to require prolonged tissue contact to exert its full antagonist effect (Barocelli *et al*., 1993). In both circular and longitudinal muscle strips from two cats, no difference in the suppression of the contraction to 1 μM carbachol by 1 μM methoctramine was found after 2 hr compared with 30 min of contact time. The pA_2 values for methoctramine and 4-DAMP were similar in the longitudinal and circular layers, whereas pirenzepine gave a slightly greater pA_2 value in longitudinal muscle than in circular muscle (table 3). The IC_{50} for a given antagonist is dependent on both agonist-receptor affinity and agonist concentration and hence cannot be directly compared with the pA_2, which is independent of these factors. However, the relative values of the $-\log IC_{50}$ (table 2) and pA_2 (table 3) are similar for methoctramine, pirenzepine and 4-DAMP.

Antagonist effects on EFS responses in circular muscle. The inhibition of nitric oxide synthase by 100 μM L-NNA was used to enhance and stabilize *on*-contractions of circular muscle as illustrated in figure 4 and previously described for human esophageal smooth muscle (Preiksaitis *et al*., 1994). Without L-NNA, EFS of circular muscle strips produced typical off-contractions, which followed EFS after a brief

TABLE 1

Effects of hexamethonium (10 μM) and N^G-nitro-L-arginine (L-NNA, 100 μM) on carbachol-mediated contraction in longitudinal and circular esophageal smooth muscles

Values represent the mean and standard error for carbachol concentration-response curves either alone (control) or after the addition of each of the drugs as indicated. Values in parentheses indicate the total number of muscle strips obtained from 2–6 cats. No value is significantly different compared with control.

Drugs	Circular Muscle		Longitudinal Muscle	
	−log EC₅₀	*% Maximum*	*−log EC₅₀*	*% Maximum*
Control	6.02 ± 0.07 (16)	100 (16)	6.69 ± 0.08 (16)	100 (16)
Hexamethonium	6.05 ± 0.08 (6)	98 ± 4 (6)	6.80 ± 0.09 (12)	97 ± 3 (12)
L-NNA	6.06 ± 0.12 (10)	106 ± 6 (10)	6.67 ± 0.09 (4)	95 ± 4 (4)
Hexamethonium + L-NNA	6.08 ± 0.10 (16)	96 ± 5 (16)	6.59 ± 0.11 (16)	98 ± 3 (16)

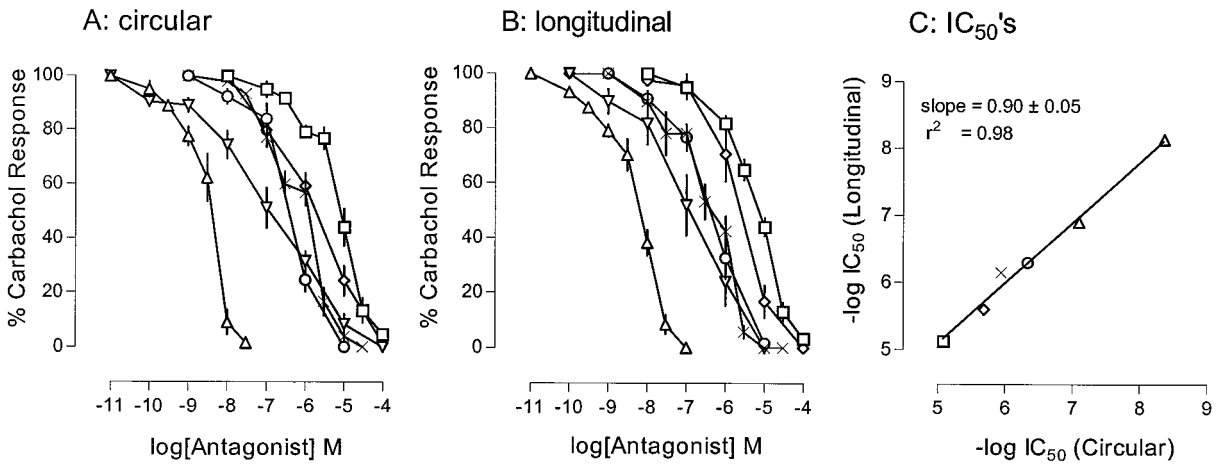

Fig. 1. Comparison of the effects of six muscarinic receptor antagonists on carbachol-mediated contractions in circular and longitudinal muscle of the esophagus. a and b, demonstrate the effects of increasing concentrations of six muscarinic antagonists (n = 4–11) on carbachol (1 μM) contraction responses in circular and longitudinal esophageal muscle, respectively. c, Comparison of the IC_{50} for each antagonist in longitudinal and circular muscle layers. The resulting line has a slope of 0.90 ± 0.05 (r^2 = .98), which is not significantly different from unity. Results for the following antagonists are shown: 4-DAMP (△), zamifenacin (▽), p-F-HHSiD (○), pirenzepine (×), AF-DX-116 (◇) and methoctramine (□).

TABLE 2

Muscarinic receptor antagonist effects on muscle contractions mediated by 1 μM carbachol and nerve-evoked responses

Negative log IC_{50} values for six antagonists were determined for contraction to a single concentration of carbachol (1 μM) in muscle strips from the longitudinal and circular muscle strips, and electrical field stimulation (EFS)-evoked, nerve-mediated *on*-contractions (100 μM N^G-nitro-L-arginine present in the bath) and *off*-contractions in circular muscle strips. IC_{50} values were determined as detailed in "Methods." These data are derived from the experiments shown in figures 1 and 5. Graphical correlations of the IC_{50} values are illustrated in figures 1C and 5C.

Antagonist	1 μM Carbachol		Nerve-evoked Contractions	
	Longitudinal	*Circular*	*On-contractions*	*Off-contractions*
Methoctramine (n)	5.13 ± 0.04 (11)	5.08 ± 0.07 (10)	5.89 ± 0.17 (8)	5.3 ± 0.15 (6)
AF-DX-116 (n)	5.64 ± 0.16 (5)	5.69 ± 0.12 (7)	6.90 ± 0.19 (3)	5.42 ± 0.10 (3)
p-F-HHSiD	6.30 ± 0.11 (6)	6.34 ± 0.08 (4)	7.56 ± 0.12 (8)	6.70 ± 0.11 (5)
Pirenzepine (n)	5.93 ± 0.06 (10)	6.23 ± 0.06 (7)	7.75 ± 0.10 (8)	6.70 ± 0.13 (4)
Zamifenacin (n)	6.76 ± 0.17 (5)	6.64 ± 0.10 (10)	8.61 ± 0.05 (4)	7.80 ± 0.13 (4)
4-DAMP (n)	8.13 ± 0.06 (9)	8.36 ± 0.09 (7)	10.52 ± 0.09 (8)	9.1 ± 0.11 (3)

latency (fig. 4B). These contractions remained stable with repetitive stimulation and were inhibited by 10 μM atropine. A small (<5%) residual, atropine-insensitive component of the *off*-contraction was noted in some preparations. After the addition of L-NNA, *off*-contractions were suppressed and replaced by *on*-contractions (fig. 4A) which also were stable with repetitive stimulation and were always completely suppressed by 10 μM atropine (fig. 4B).

The effects of the 6 muscarinic receptor antagonists on EFS responses in circular muscle were examined and compared to the effects on carbachol-induced contractions. The antagonists demonstrated similar relative selectivities for suppressing off-contractions (fig. 5A) and on-contractions (determined in the presence of L-NNA, fig. 5B). As shown in figure 5C and table 2, the $-\log IC_{50}$ for the effect of these 6 antagonists on carbachol-mediated responses correlated linearly with both on-contractions and off-contractions yielding slope values of 1.3 ± 0.1 (r^2 = .97) and 1.2 ± 0.2 (r^2 = .89), respectively, neither of which differs significantly from unity.

Identification of m_2 and m_3 receptor mRNA by RT-PCR. To further explore the potential roles of M_2 and M_3 receptor subtypes in the cat esophageal smooth muscle, RT-PCR was used to identify mRNA species encoding these two receptor types in RNA isolated from esophageal muscle samples, which included both longitudinal and circular layers. Since the gene sequences for the cat muscarinic receptor subtypes were previously unknown, PCR primers based on the known human gene sequences were used. Messenger RNA for the m_3 receptor could be readily identified in 3 of 3 cats, as well as terminal ileum which served as control (fig. 6). The level of expression of m_2 mRNA was not detected in the same 3 cats despite using optimum conditions to amplify the m_2 sequence as determined for cat heart tissue, which served as the positive control for the m_2 receptor. The identity of the PCR products for both receptor subtypes was verified by sequence analysis using the m_2 product from myocardial tissue and the m_3 product from esophageal body (fig. 7). Comparison with the known sequences for the human m_2 and m_3 genes demonstrated a high degree of nucleotide sequence homology for the amplified portions cloned from cat tissues (93% and 89%, respectively). The upstream and downstream PCR primer sequences selected were identical for the cat and the human genes (fig. 7). Figure 8 shows the comparison of the corresponding amino acid sequences for the human and cat m_2 and m_3 receptors.

Discussion

Despite the large number of muscarinic receptor agonists and antagonists available, none has sufficient selectivity for one receptor subtype over all others to allow the unequivocal pairing of receptor type and pharmacological response. However the order and pattern of selectivities of a group of antagonists for the receptor can provide sufficient grounds for

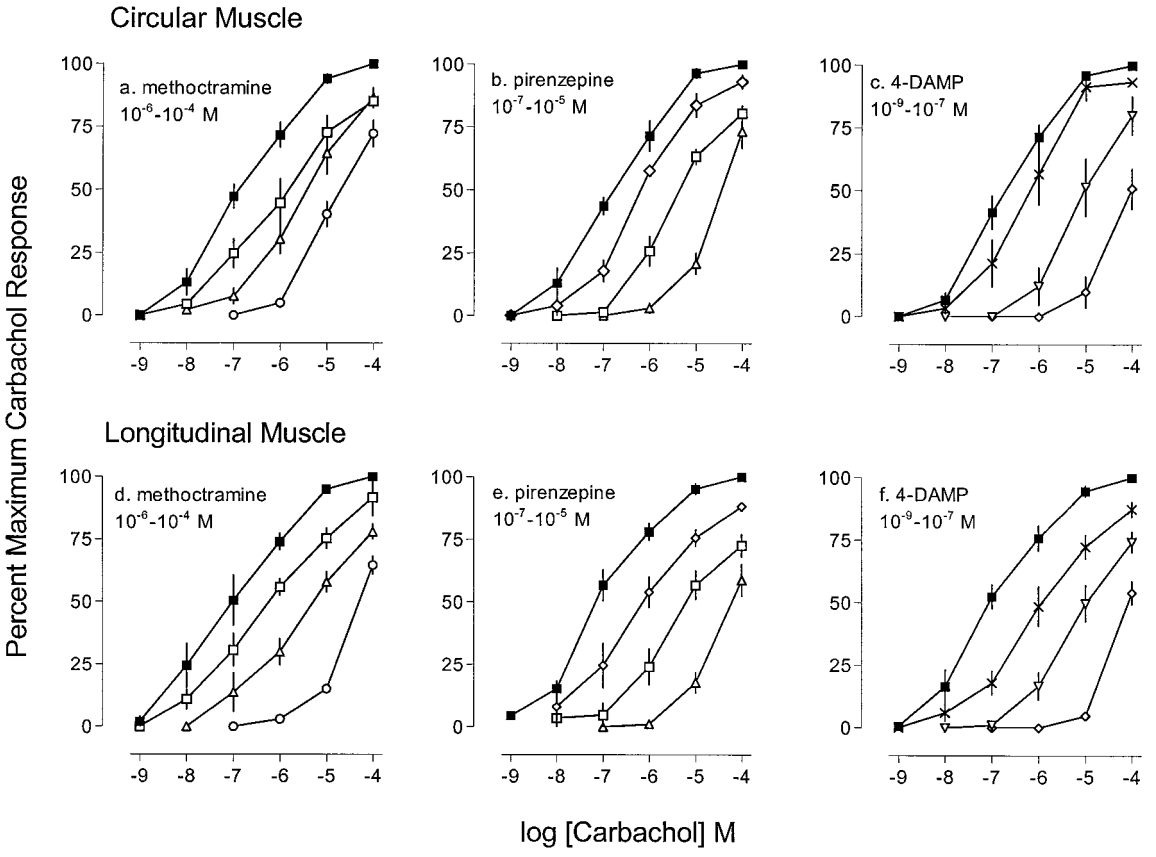

Fig. 2. Antagonism of carbachol-mediated contractions in circular (a–c) and longitudinal (d–f) muscles from the cat esophagus by methoctramine (a, d), pirenzepine (b, e) and 4-DAMP (c, f). Symbols represent the following antagonist concentrations: (■) none, (×) 10^{-9} M, (▽) 10^{-8} M, (◇) 10^{-7} M, (□) 10^{-6} M, (△) 10^{-5} M and (○) 10^{-4} M. Values shown represent the means for 3 to 6 muscle strips obtained from 9 cats.

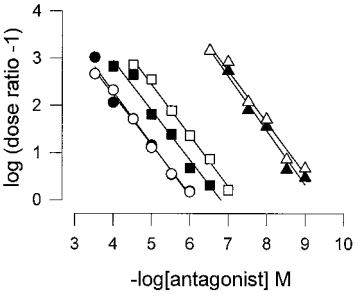

Fig. 3. Schild plots of methoctramine (○, ●, n = 5), pirenzepine (□, ■, n = 4) and 4-DAMP (△, ▲, n = 4) antagonism of carbachol-mediated responses in longitudinal (open symbols) and circular (closed symbols) smooth muscles from the cat esophagus. Lines shown are the best fit by least-squares linear regression. The resulting slopes and x-intercepts (pA_2 values) are summarized in table 3.

the classification of a given response (Eglen *et al*., 1996a; Caulfield, 1993; Hulme *et al*., 1990). By applying this strategy, we were able to characterize the muscarinic receptor subtype in cat esophageal smooth muscle. Our findings can be summarized as follows: (1) Carbachol-induced contraction of smooth muscle in both the longitudinal and circular layer of the esophageal body demonstrates a pattern of antagonist selectivity best represented by the M_3 receptor subtype. (2) The pattern of antagonist selectivity for inhibiting both on- and off-type intrinsic nerve-mediated contractions also is characteristic of the M_3 receptor subtype. Thus, both nerve-mediated and carbachol-induced contractions of the cat esophageal body muscles result through activation of M_3 receptors. (3) These observations are further corroborated by RT-PCR data, which show that mRNA encoding the m_3 receptor is readily detected in the cat esophageal body smooth muscle, whereas mRNA for the m_2 receptor is not.

Both the longitudinal and circular muscles of the esophagus showed similar relative antagonist selectivity for carbachol-mediated contractions. The high selectivity of 4-DAMP, intermediate selectivity of pirenzepine, and low selectivity of methoctramine in antagonizing carbachol-mediated responses in both muscle layers constitute compelling evidence that contraction is mediated by M_3 receptors. The pA_2 values obtained are similar to those found in functional studies of the guinea pig terminal ileum (Eltze and Figala, 1988; Eglen *et al*., 1992a; Eglen and Harris, 1993), a typical M_3-mediated response, and for M_3-mediated contractile responses in other smooth muscle types (Shi and Sarna, 1997; Eglen *et al*., 1996a; Caulfield, 1993). Furthermore, the pA_2 values for these three antagonists correspond to the $-\log K_i$ determined by radioligand binding studies on the M_3 receptor in native tissues (Hulme *et al*., 1990) and using heterologous expression of the human M_3 receptor gene product (Eglen *et al*., 1996a). In both muscle layers, these three drugs showed competitive antagonism of the carbachol responses, producing parallel rightward shifts in the dose response curves, and linear Schild plots with slopes not significantly different from unity. Since 4-DAMP has poor selectivity for M_3 over M_1 receptors, and only a ~9- to 10-fold selectivity ratio of M_3 over M_2 receptors (Dorje *et al*., 1991), our conclusion depends substantially on the observation that methoctramine (M_2/M_4

TABLE 3
Schild analysis of inhibition of carbachol-mediated contractions in longitudinal and circular esophageal muscle by selective muscarinic antagonists

Antagonist	Circular Muscle		Longitudinal Muscle	
	pA_2	*Slope*	pA_2	*Slope*
Methoctramine $n = 5$	6.04 ± 0.11	−1.10 ± 0.07	6.10 ± 0.06	−1.06 ± 0.04
Pirenzepine $n = 4$	6.79 ± 0.09	−1.05 ± 0.06	7.26 ± 0.07[a]	−1.08 ± 0.04
4-DAMP $n = 4$	9.25 ± 0.12	−1.15 ± 0.08	9.46 ± 0.14	−1.09 ± 0.08

[a] Significantly different when compared with corresponding value for circular muscle (P = .007).

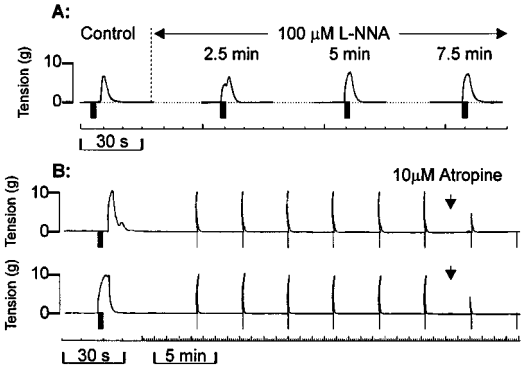

Fig. 4. Effect of L-NNA and atropine on electrical field stimulation (EFS) responses in circular esophageal smooth muscle. Each tracing is from a single smooth muscle strip, with the heavy bar just below the tracing indicating the time of application of the EFS train. a, L-NNA (100 μM) caused the suppression of the off-contraction (which follows the cessation of the EFS train) and the emergence of the intra-stimulus on-type contraction. b, Repetitive EFS with 3-sec trains applied every 180 sec caused reproducible off-contractions (upper trace of b) and on-contractions in the presence of 100 μM L-NNA (lower trace of b). Both on- and off-contractions were antagonized completely by 10 μM atropine.

selective) poorly antagonized carbachol responses, whereas pirenzepine (M_1 selective) had an intermediate effect. The pA_2 value we found for methoctramine is too low to consider either a M_2- or M_4-mediated response (Eglen *et al*., 1996a). Schild slopes for methoctramine that deviate significantly from unity have been observed in some preparations, suggesting inadequate tissue equilibration time (Barocelli *et al*., 1993) or a noncompetitive, allosteric effect of this antagonist (Eglen *et al*., 1988). In the present study, exposure to methoctramine for 2 hr did not result in any difference in antagonist effectiveness compared to 30 min. Thus, we found no evidence for disequilibrium or allosteric effects of methoctramine in the present study.

The pA_2 value we report for pirenzepine in the longitudinal muscle (7.26) is significantly greater than that found in the circular muscle (6.79) and is slightly greater than might be expected based on other M_3-mediated responses (6.7–7.1, Caufield, 1993). Nonetheless, our conclusion that the M_3 receptor mediates this response in the longitudinal muscle is valid for several reasons: (1) The pA_2 value we calculate for all three antagonists fits the pattern expected for a M_3-mediated response better than for any other subtype (Eglen *et al*., 1996a; Caulfield, 1993). (2) A pA_2 value for pirenzepine in the range of 8.1 to 8.5 would be expected for a M_1-mediated response (Caulfield, 1993). (3) A similar pA_2 value of 7.23 was reported for the M_3-mediated contraction of human colonic smooth muscle (Kerr *et al*., 1995). (4) Finally, M_1 receptors are present on enteric ganglia, but have not been localized to gastrointestinal smooth muscle cells (Goyal, 1989; Eglen *et al*., 1996a). Investigation of the possible mechanisms underlying the difference in the effectiveness of pirenzepine or the potency of carbachol in longitudinal and circular muscles is beyond the scope of this study.

The good linear correlation between the $-\log IC_{50}$ values for the six muscarinic antagonists against 1 μM carbachol-mediated contractions and both on- and off-type nerve-mediated contractions in the circular muscle layer leads us to conclude that nerve-mediated responses in this tissue also are mediated predominantly by M_3 receptors. On- and off-contractions occur *via* different mechanisms, the latter being dependent on the action of noncholinergic, nonadrenergic inhibitory nerves, the main mediator being nitric oxide (Preiksaitis *et al*., 1994; Murray *et al*., 1991). The mechanism by which cholinergic and noncholinergic nerves interact to bring about the off-contraction is unknown. Differences in the mechanism of on- and off-contractions might include the amounts of acetylcholine released. We speculate that these factors could explain why the correlation between antagonist effects on carbachol-responses and both types of nerve-mediated contractions results in distinct parallel lines, with slopes not different from unity. In both cases, pirenzepine appears to have a greater effect on nerve-mediated responses than carbachol contractions possibly due to a selective interaction of pirenzepine with M_1 receptors present on enteric ganglia, which may additionally modulate the nerve-mediated responses (Gilbert *et al*., 1984). Our conclusion that nerve-mediated (acetylcholine) contractions and carbachol responses are due mainly to activation of M_3 receptors is based on the assumption that acetylcholine and carbachol display similar selectivity for muscarinic receptor subtypes. In the absence of evidence to the contrary, this assumption can be justified since carbachol is a close analogue of acetylcholine and the minor structural difference does not involve the key site for interaction with the receptor (Hulme *et al*., 1990).

Zamifenacin and *p*-F-HHSiD, two additional M_3-selective antagonists, also were similarly effective in inhibiting nerve- and carbachol-mediated contractions. Schild analysis of these antagonists was not carried out in the present study, however both antagonists were less effective inhibitors than could be anticipated based on previous studies on other tissues (Eglen *et al*., 1990; Watson *et al*., 1995; Barlow *et al*., 1995; Feifel *et al*., 1990). In the present study, the IC_{50} value obtained for the carbachol response in circular muscle was 100-fold less for *p*-F-HHSiD compared with 4-DAMP. Low pA_2 values for *p*-F-HHSiD previously have been noted by others and this appears to be dependent on the preparation used: the pA_2 values for *p*-F-HHSiD and 4-DAMP differ by ~100-fold in the guinea pig trachea (Eglen *et al*., 1990). A recent study which demonstrated that carbachol responses in human colonic muscle showed an antagonist profile most consistent with the M_3 receptor, found a 45- to 300-fold difference in the pA_2 values for *p*-F-HHSiD and 4-DAMP in the longitudinal and circular muscle layers, respectively

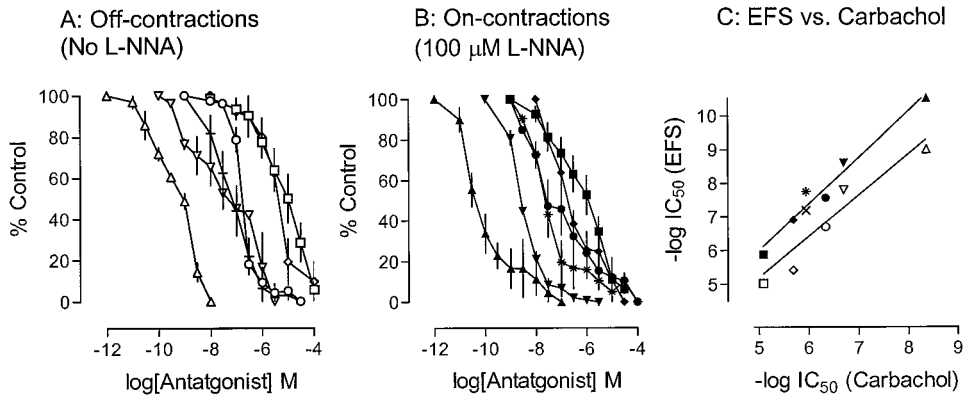

Fig. 5. Effects of increasing concentrations of muscarinic antagonists on EFS-mediated responses in circular esophageal muscle. a, Effect on off-contractions (no L-NNA present). b, Effect on on-contractions (in the presence of 100 μM L-NNA). c, Comparison of IC_{50}'s for effects of muscarinic antagonists on EFS-mediated on-contractions (closed symbols and ×) and off-contractions (open symbols and +) to carbachol-mediated responses in circular esophageal muscle. Both comparisons yielded straight lines with slopes of 1.2 ± 0.2 (r^2 = .89) for off-contractions and 1.3 ± 0.1 (r^2 = .97) for on-contractions, neither of which differs significantly from unity. Results for the following antagonists are shown: 4-DAMP (△, ▲), zamifenacin (▽, ▼), p-F-HHSiD (○, ●), pirenzepine (×, +), AF-DX-116 (◇, ◆) and methoctramine (□, ■). Each data point represents the mean of n = 3–8 cats.

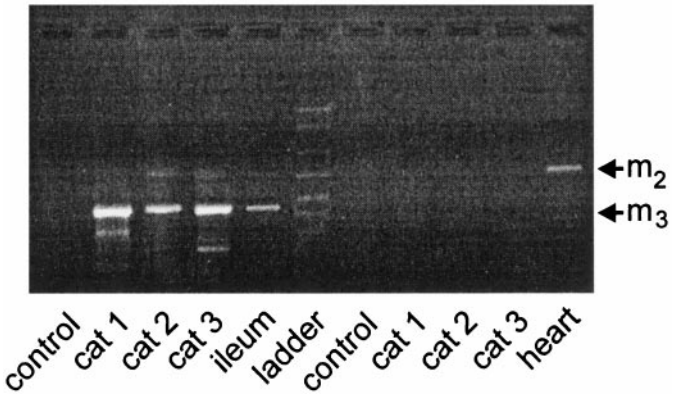

Fig. 6. Identification of M_2 and M_3 receptor messenger RNA in esophageal smooth muscle, heart and terminal ileum by RT-PCR. Messenger RNA coding for the M_3 receptor was readily identified in esophageal tissues from 3 cats and terminal ileum which served as a positive control. In contrast, mRNA for the M_2 receptor was not detected in any esophageal specimen, but was readily detected in heart tissue which served as a positive control. Control lanes shown are the products of the PCR reaction without cDNA present. The ladder shows bands corresponding (top to bottom) to 2000, 1500, 1000, 700, 500, 400 and 300 kb.

Cat m_2

GGTCAGCAATGCCTCAGTTATGAATCTGCTCATTATCAGCTTTGATAGGTACT
TCTGTGTCACCAAACCGCTCACCTACCCAGTCAAGCGGACCACGAAAATGG
CAGGTATGATGATTGCAGCTGCCTGGGTCCTCTCCTTTATCCTGTGGGCCCC
AGCCATTCTCTTCTGGCAGTTCATCGTAGGGGTGAGGACTGTAAAGGATGG
GGAATGCTACATTCAGTTTTTCTCCAATGCCGCTGTCACCTTTGGTACCGCC
ATTGCAGCCTTCTATTTGCCTGTGATCATCATGACTGTGCTATACTGGCACAT
ATCCCGAGCCAGCAAGAGCAGGATAAAGAAGGACTAGAAGGAGCCTGTGGC
CAACCAAGAGCCAGTTTCTCCAAGTCTGGTACAAGGAAGGATAGTGAGGCC
AAACAACAACACTACACCTGGCAGTGACGGTAGCCTGGAGCACAACAAAATC
CAGAATGGCAAAGCCCCCAAAGATGCTGTGACTGAAAACTGTGTCCAGGGA
GAGGAGAAGGAGAGCTCCAATGATTCCACCTCAGTCAGTGCGGTAGCCTCT
AATATGAGAGATGATGAAATAACCCAGGATGAGAACACAGTCTCCACTTCCC
TGGGCCACTCCAAAGATGAGAACTCGAAGCAGACGTGCATCAGAATTGGCA
CCAAG

Cat m_3

TGATGATCGGTCTGGCTTGGGTCATCTCCTTCATCCTTTGGGCCCCCGCCAT
CTTGTTCTGGCAGTACTTTGTTGGGAAGAGAACTGTGCCCCCAGGGGAGTG
CTTCATTCAGTTCCTCAGCGAGCCCACCATCACCTTCGGCACGGCCATCGCT
GCCTTCTACATGCCTGTCACCATCATAGCTATTTTATACTGGAGGATCTACAA
GGAAACTGAAAAACGCACCAAAGAGCTCGCCTGCCTGCAAGCCTCGGGGAC
AGAAGCAGAGGCGGAAAACTTTGTCCACCCCACGGGCAGCTCTCGAAGCTG
CAGCAGCTATGAGCTTCAACAGCAAAGTATGAAACGCTCGGCCAGGAGGAA
GTACGGACGCTGTCACTTCTGGTTCGCCACCAAGAGTTGGAAGCCCAGTGC
CGAGCAGATGGACCAAGACCACAGCAGCA

Fig. 7. Partial sequences for the cat m_2 and cat m_3 receptor genes.

(Kerr *et al*., 1995). Similarly, a range of pA_2 values has been observed for zamifenacin antagonism of M_3 responses depending on the smooth muscle type studied (Watson *et al*., 1995). The pA_2 for zamifenacin in guinea pig terminal ileum was 9.3, similar to the pA_2 for 4-DAMP in the same preparation, but ~50-fold less effective for guinea pig urinary bladder (pA_2 = 7.6). In the present experiments, zamifenacin was ~20-fold less effective than 4-DAMP, indicating a more modest antagonist effect in cat esophageal smooth muscle.

In vivo, pirenzepine has little effect on esophageal peristalsis in animal models (Gilbert and Dodds, 1986; Blank *et al*., 1989). In contrast, 4-DAMP completely blocks peristalsis in the cat and significantly decreases contraction amplitude in the opossum (Gilbert and Dodds, 1986; Blank *et al*., 1989). This species difference could be anticipated, since an atropine-resistant contribution to the off-contraction accounts for ~60% in the distal esophagus of the opossum (Crist *et al*., 1984), while previous studies (Behar *et al*., 1989; Leander *et al*., 1982) and our findings indicate that the off-contraction in the cat is highly sensitive to atropine. In the opossum lower esophageal sphincter, 4-DAMP potently inhibited agonist-mediated *in vivo* contraction (Gilbert *et al*., 1984). These earlier studies were interpreted to indicate that contractions in circular muscle of the esophagus and lower esophageal sphincter are M_2-mediated. Since it is now recognized that 4-DAMP potently antagonizes the M_3 receptor, it is more likely that the receptor characterized in the above reports is the M_3 subtype as demonstrated here. The present study and those cited above concern the muscularis propria of the esophagus. The M_3 muscarinic receptor subtype also mediates cholinergic responses in the musclularis mucosae of the esophagus in the rat, guinea pig, and rabbit (Hatakeyama *et al*., 1995; Thomas and Ehlert, 1996; Eglen *et al*., 1996b).

In studies on smooth muscle cells isolated from the circular layer of the muscularis propria of the cat esophageal body and lower esophageal sphincter, Sohn *et al*. (1993) concluded that cell shortening was mediated by the M_3 receptor in the sphincter and the M_2 receptor in the esophageal body. They observed a low pA_2 value for *p*-F-HHSiD (6.78) and an unusually high pA_2 value for methoctramine (9.05) in cells from

```
                                                                                                |----------M-I------
hm2     --------------- --------------- --------------- MNNSTNSSNNSLALT SPYKTFEVVFIVLVA GSLSLVTIIGNILVM     45
cm2     --------------- --------------- --------------- --------------- --------------- ---------------      0
hm3     MTLHNNSTTSPLFPN ISSSWIHSPSDAGLP PGTVTHFGSYNVSRA AGNFSSPDGTTDDPL GGHTVWQVVFIAFLT GILALVTIIGNILVI     90
cm3     --------------- --------------- --------------- --------------- --------------- ---------------      0

        ---|    |----------M-II-------------|     |----------M-III---------|
hm2     VSIKVNRHLQTVNNY FLFSLACADLIIGVF SMNLYTLYTVIGYWP LGPVVCDLWLALDYV VSNASVMNLLIISFD RYFCVTKPLTYPVKR    135
cm2     --------------- --------------- --------------- --------------- VSNASVMNLLIISFD RYFCVTKPLTYPVKR     30
hm3     VSFKVNKQLKTVNNY FLLSLACADLIIGVI SMNLFTTYIIMNRWA LGNLACDLWLAIDYV ASNASVMNLLVISFD RYFSITRPLTYRAKR    180
cm3     --------------- --------------- --------------- --------------- --------------- ---------------      0

            |-----------M-IV---------|                     |----------M-V----------|
hm2     TTKMAGMMIAAAWVL SFILWAPAILFWQFI VGVRTVEDGECYIQF FSNAAVTFGTAIAAF YLPVIIMTVLYWHIS RASKSRIKKDKKEPV    225
cm2     TTKMAGMMIAAAWVL SFILWAPAILFWQFI VGVRTVKDGECYIQF FSNAAVTFGTAIAAF YLPVIIMTVLYWHIS RASKSRIKKD-KEPV    119
hm3     TTKRAGVMIGLAWVI SFVLWAPAILFWQYF VGKRTVPPGECFIQF LSEPTITFGTAIAAF YMPVTIMTILYWRIY KETEKRTKELAGLQA    270
cm3     -------MIGLAWVI SFILWAPAILFWQYF VGKRTVPPGECFIQF LSEPTITFGTAIAAF YMPVTIIAILYWRIY KETEKRTKELACLQA     83

hm2     ANQDPVSPSLVQGRI VKPNNNNMPSSDDGL EHN-KIQNGKAPRDP VTENCVQGEEKESSN DSTSVSAVASNMR-- ---------------    297
cm2     ANQEPVSPSLVQGRI VRPNNNTTPGSDGSL EHN-KIQNGKAPKDA VTENCVQGEEKESSN DSTSVSAVASNMR-- ---------------    191
hm3     SGTEAETENFVHPTG SSRSCSSYELQQQSM KRSNRRKYGRCHFWF TTKSWKPSSEQMDQD HSSSDSWNNNDAAAS LENSASSDEEDIGSE    360
cm3     SGTEAEAENFVHPTG SSRSCSSYELQQQSM KRSARRKYGRCHFWF ATKSWKPSAEQMDQD HSS------------ ---------------    146

hm2     --------------- -----------DDEI TQDENTVSTSLGHSK DENSKQTCIRIGTKT PKSDSCTPTNTTVEV VGSSGQNG-------    354
cm2     --------------- -----------DDEI TQDENTVSTSLGHSK DENSKQTCIRIGTK- ---------------- ----------------    224
hm3     TRAIYSIVLKLPGHS TILNSTKLPSSDNLQ VPEEELGMVDLERKA DKLQAQKSVDDGGSF PKSFSKLPIQLESAV DTAKTSDVNSSVGKS    450
cm3     --------------- --------------- --------------- ---------------- ---------------- ----------------    146

                                                |----------M-VI----------|       |----------M-VII-
hm2     --------DEKQNIV ARKIVKMTKQPAKKK PPPSREKKVTRTILA ILLAFIITWAPYNVM VLINTFCAPCIPNTV WTIGYWLCYINSTIN    436
cm2     --------------- --------------- --------------- ---------------- ---------------- ----------------    224
hm3     TATLPLSFKEATLAK RFALKTRSQITKRKR MSLVKEKKAAQTLSA ILLAFIITWTPYNIM VLVNTFCDSCIPKTF WNLGYWLCYINSTVN    540
cm3     --------------- --------------- --------------- ---------------- ---------------- ----------------    146

        ----|
hm2     PACYALCNATFKKTF KHLLMCHYKNIGATR ---------------- -----    466
cm2     --------------- --------------- ---------------- -----    224
hm3     PVCYALCNKTFRTTF KMLLLCQCDKKKRRK QQYQQRQSVIFHKRA PEQAL    590
cm3     --------------- --------------- ---------------- -----    146
```

Fig. 8. Comparison of the known amino acid sequences for the human m_2 (hm2) and m_3 (hm3) receptors and the portions of the cat m_2 (cm2) and m_3 (cm3) receptors amplified by RT-PCR in the present study. The approximate positions of the seven transmembrane-spanning regions (MI-MVII) are shown in bold type above the amino acid sequences. The portions of the human sequences corresponding to the RT-PCR primer sets used are shown in bold type.

the esophageal body, and a low pA_2 value for methoctramine (6.53) and a high pA_2 value for *p*-F-HHSiD (8.61) in cells from the sphincter. Our conclusion that cholinergic contractions in intact muscle strips from the cat esophageal body are mediated by a M_3 receptor mechanism are at odds with the above findings. We were unable to detect mRNA for the M_2 receptor in the cat esophagus, although the significance of this finding must be interpreted cautiously since we have not assayed for the presence of M_2 receptors *per se*; the mRNA content of a tissue may not consistently reflect receptor expression since other factors such as receptor turnover may be important. In many smooth muscles, the M_2 receptor population dominates, while the less plentiful M_3 receptor mediates the contraction response (Eglen *et al*., 1996a). Although, our experiments show a dominant role for the M_3 receptor in mediating functional cholinergic responses, we cannot rule out a contribution by the M_2 receptor subtype. As yet there is no explanation for the differing contribution of M_2 and M_3 receptors in intact muscle strips compared with muscle cells isolated by enzymatic digestion (Sohn *et al*., 1993).

The M_3 receptor preferentially activates phospholipase Cβ, stimulating the production of inositol-1,4,5 trisphosphate, which triggers the release of calcium from intracellular stores (Hulme *et al*., 1990; Eglen *et al*., 1996a). In the cat, a significant increase in inositol 1,4,5 trisphosphate in response to acetylcholine stimulation was observed in cells isolated from the circular muscle of the lower esophageal sphincter but not the esophageal body (Sohn *et al*., 1993). Previous studies on cat and opossum smooth muscle strips showed that the cholinergic contraction is dependent on extracellular calcium (De Carle *et al*., 1977; Biancani *et al*., 1987). On the other hand, patch-clamp experiments using cells isolated from the cat esophageal muscle reported by Sims *et al*. (1990) have demonstrated cholinergic activation of potassium current, indicative of the release of calcium from intracellular stores. Moreover, Kirber and Biancani (1996) recently have found direct evidence for the release of intracellular calcium accompanying acetylcholine-induced contraction of these cells. These latter observations are compatible with a M_3-mediated activation of the phospholipase Cβ pathway. Finally, recent studies on human esophageal muscle, from our laboratory, are consistent with a dominant functional role for the M_3 receptor and a contractile mechanism, which also involves the mobilization of intracellular calcium stores (Sims *et al*., 1997; Preiksaitis *et al*., 1996). Although the present data clearly support a major role for the M_3 receptor in the cholinergic response of the cat esophageal smooth muscle, further studies are required to clarify the postreceptor mechanisms involved.

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