0031-6997/96/4804-0531$03.00/0
PHARMACOLOGICAL REVIEWS
Vol. 48, No. 4

Muscarinic receptor subtypes and smooth muscle function

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I. Introduction

The autonomic nervous system consists of various neural pathways associated with ganglionic synapses residing outside of the central nervous system. One part of the autonomic nervous system, the parasympathetic nervous system, is composed of neurons arising from the brain stem and sacral spinal cord (see Buckley and Caulfield, 1992 for review). Acetylcholine is the principal neurotransmitter of the parasympathetic nervous system, being released at both ganglionic synapses and at post-ganglionic neuro-effector junctions (fig. 1). Although the release of co-transmitters often is integrated with the actions of acetylcholine at target organs, this aspect will not be discussed because of space limitations (see Lundberg, 1996 for review). The intracellular effects of acetylcholine are mediated by activation of nicotinic and muscarinic cholinergic receptors (Dale, 1914, 1933; see Burgen, 1995 for review). These receptors are, themselves, composed of multiple subtypes, with differing structure, pharmacology and distribution (Hosey, 1992). The muscarinic cholinergic receptor family is composed of five subtypes, encoded by five distinct, but related, genes (Hulme et al., 1990).

Classically, muscarinic receptors were operationally defined on the basis of selective agonism by muscarine and antagonism by atropine (see Burgen, 1995, for review). In retrospect, these naturally occurring compounds were uniquely selective for these receptors, inasmuch as subsequent attempts to identify novel compounds with equivalent selectivity for a single muscarinic receptor subtype have failed (Caulfield, 1993). A major aim of current research in this area consequently lies in the identification of selective ligands for each of these subtypes. In terms of agonists that discriminate on the basis of affinity, this aim has not been achieved (Caulfield, 1993), but significant progress is being made in the development of functionally selective compounds (see Eglen and Watson, 1996 for review). Several advances have been made in the identification of selective antagonists (Eglen and Watson, 1996). Together, these compounds will prove useful as both therapeutics and tools to operationally define muscarinic receptor subtypes.

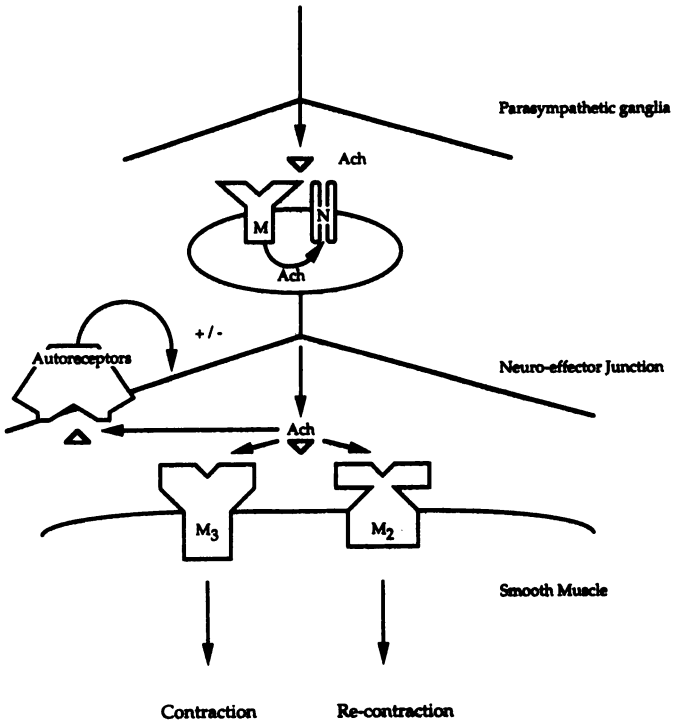

FIG. 1. Schematic representation of the parasympathetic innervation in smooth muscle. M, muscarinic receptor; N, nicotinic receptor; Ach, acetylcholine.

Historically, isolated smooth muscle tissues have played an important role in muscarinic receptor research, because of their ease of preparation and the magnitude and clarity of the contractile response to cholinergic agonists. Indeed, until the routine use of radioligand binding assays, the identification of novel muscarinic receptor agonists and antagonists relied mostly on smooth muscle bioassays, including guinea pig or rat isolated ileum or rabbit isolated jejunum (see Bebbington and Brimblecombe, 1965 for review). These tissues allowed several structure activity relationships to be developed for agonists and antagonists (e.g., Abramson et al., 1969; Barlow et al., 1972, 1976; Ringdahl and Jenden, 1983; Ringdahl, 1985; Grana et al., 1987). Moreover, early radioligand binding studies at muscarinic receptors also used membranes isolated from gastrointestinal smooth muscle (e.g., Paton and Rang, 1965; Yamamura and Snyder, 1974; Burgen et al., 1974). An assumption in these early studies was that a single muscarinic receptor subtype mediated smooth muscle contractile responses, and the ligands available labeled a homogeneous population of sites. It is now evident from binding and other studies with subtype-selective antagonists that smooth muscles express several muscarinic receptor subtypes (Giraldo et al., 1987, 1988; Michel and Whiting, 1987, 1988; Baudiere et al., 1987; Roffel et al., 1988), each of which contribute to the functional response (see Ehlert and Thomas, 1995; Eglen et al., 1994a, b for recent reviews).

Systemic injection of acetylcholine increases muscular tone and movement of the gut and urinary bladder, increases bronchiolar and pupillary constriction, and generally increases vasodilatation, leading to hypotension. The present paper reviews muscarinic receptor subtypes in the context of these different tissues; specifically, gastrointestinal, genitourinary, respiratory, ocular and vascular tissues. The primary aim is to assess recent studies on the role of heterogeneous muscarinic receptor populations in regulating smooth muscle function. This subject has not been addressed in depth before, although brief summaries have been published (Eglen et al., 1994b). The focus of the review will center on post-junctional muscarinic receptors, because a recent

review of pre-junctional muscarinic receptors has been published previously (Grimm et al., 1994). Related aspects of muscarinic receptor research, including the molecular biology, biochemistry and medicinal chemistry of muscarinic receptors and their ligands, also have been extensively reviewed (Eglen and Whiting, 1986; Mitchelson, 1988; Hulme et al., 1990; Hosey, 1992; Caulfield, 1993; Moltzen and Bjornhólm, 1995; Jaen and Davis, 1994; Eglen and Watson, 1996). The bibliographies cited in these papers should be consulted for additional information.

II. Classification of Muscarinic Receptor Subtypes

A. Sequence and Predicted Structure

Neurohormonal receptors are classified by integrating operational i.e., pharmacological, transductional and structural data (Kenakin et al., 1992). The most fundamental criteria for classification is the primary amino acid sequence, preferably of human gene products (Vanhoutte et al., 1996). Muscarinic receptors are encoded by five distinct, but homologous, intronless genes (table 1). The expressed proteins, conforming to the archetypal structure of guanine nucleotide binding protein (G protein) coupled receptors, are highly homologous within the seven membrane spanning domains. Muscarinic receptors possess a large cytoplasmic loop between the fifth and sixth membrane spanning region, which is highly divergent between subtypes and considered to be principally responsible for coupling to G proteins (Felder, 1995). In recombinant systems, transfected muscarinic receptor gene products, denoted m1, m2, m3, m4 and m5 receptors, broadly correspond to those receptors defined on pharmacological criterion i.e., M_1, M_2, M_3 and M_4 receptors (Hulme et al., 1990; Dorje et al., 1991b; Dong et al., 1995). The m5 gene product currently lacks a clear endogenous correlate and will be designated in this review by the lower case appellation (Vanhoutte et al., 1996). At high expression levels, muscarinic receptors promiscuously couple to several signaling systems (Fukuda et al., 1989). In general, however, muscarinic M_1, M_3 and m5 receptors preferentially couple to mobilization of intracellular calcium, by augmentation of phosphoinositide hydrolysis, whereas activation of muscarinic M_2 and M_4 receptors inhibit adenylyl cyclase activity (see Felder, 1995, for review; table 1). Other signaling systems have been identified, albeit less extensively defined, including activation of phospholipases A_2 and D, tyrosine kinase, and calcium or potassium ion influx (Felder, 1995). It is evident, therefore, that classification of muscarinic receptor subtypes on the basis of signal transduction pathways per se is inadequate (Caulfield, 1993).

B. Pharmacology

Pharmacological studies of muscarinic receptor subtypes have classified many responses of smooth muscle studied to date. Characterization is undertaken by determining the affinities (functional studies, pK_B; radioligand binding studies, pK_i) of a small number of key antagonists for the receptor (fig. 2). In functional studies, null methods are generally used to obtain these values, in which it is assumed that the relationship between occupancy of the receptor by agonist and tissue response is equivalent in both the absence and presence of antagonist.

The antagonists (tables 2 and 3) extensively used in characterizing smooth muscle responses (tables 4-8) include atropine (non-selective), pirenzepine (M_1 selective; Hammer et al., 1980), methoctramine (M_2/M_4 selective; Melchiorre et al., 1987), 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) (M_1/M_3 selective; Barlow et al., 1976; Brown et al., 1980) *para*-fluorohexahydrosiladifenidol (*p*-F-HHSiD; M_3 selective; Lambrecht et al., 1988; 1989a, b) and himbacine (M_2/M_4 selective; Gilani

[b] Abbreviations: 4-DAMP, 4-diphenylacetoxy-N-methyl piperidine methiodide; 4-DAMP mustard, 4-diphenyl-N-(2-chloroethyl)-piperidine; *p*-F-HHSiD, *para*-fluorohexahydrosiladifenidol; RT-PCR, reverse transcript-polymerase chain reaction; pEC_{50}, $-logEC_{50}$; mRNA, messenger ribonucleic acid; 3-CP-4-DAP, N-(3-hydroxypropyl)-4-piperidinyl diphenylacetate; cAMP, cyclic adenosine monophosphate; $InsP_3$, (1,4,5)-triphosphate; DG, diacylglycerol; 5-HT, 5-hydroxytryptamine; EpDRF, epithelium derived relaxant factor; QNB, quinuclidinyl benzylate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PKC, protein kinase C; PLC, phospholipase C; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; NMS, N-methylscopolamine.

TABLE 1
Characteristics of muscarinic receptor subtypes

Nomenclature	M_1	M_2	M_3	M_4
Receptor gene	m1	m2	m3	m4
Structure	7TM	7TM	7TM	7TM
human	460aa	466aa	590aa	479aa
mouse	460aa	—	—	479aa
rat	460aa	466aa	589aa	478aa
porcine	460aa	466aa	590aa	479aa
Intracellular messenger	$InsP_3$/DAG	cAMP/K^+ channels	$InsP_3$/DAG	cAMP

TM, predicted number of transmembrane spanning domains; aa, amino acid residues; $InsP_3$/DAG, (mobilization); cAMP, (reduction). A fifth gene, m5, has been cloned, but a functional correlate has not been unambiguously demonstrated.

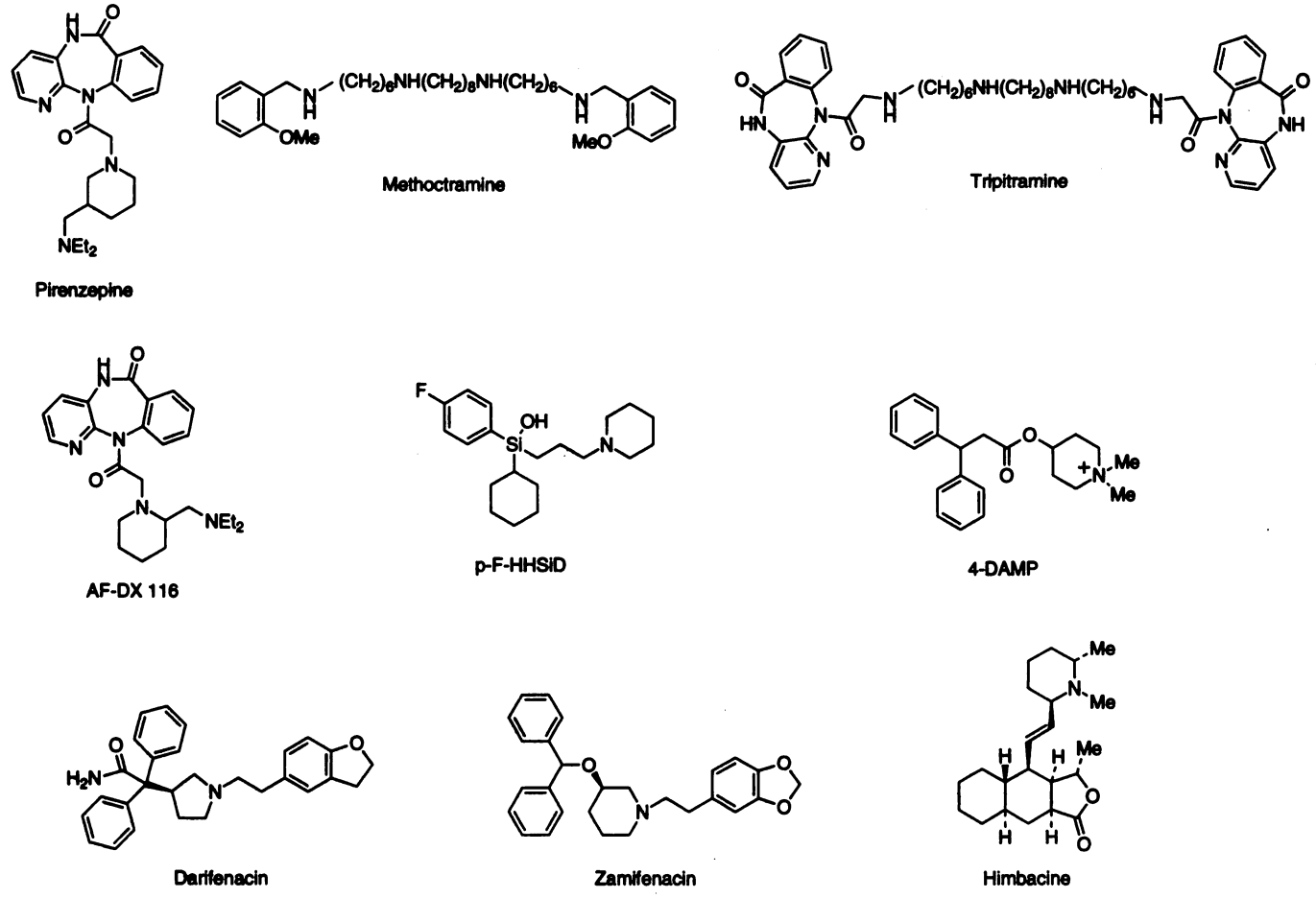

FIG. 2. Structures of key antagonists used in the pharmacological characterization of muscarinic receptor subtypes.

TABLE 2
Pharmacological characterization of muscarinic receptors

Antagonist	M_1	M_2	M_3	M_4	m5
4-DAMP	8.6 (9.2)	7.8 (8.1)	9.1 (9.3)	ND (8.4)	(8.9)
darifenacin	7.9 (7.8)	6.9 (7.0)	9.4 (8.9)	ND (7.7)	(8.1)
himbacine	7.2 (6.6)	8.5 (7.9)	7.6 (6.9)	8.8 (7.4)	(6.1)
methoctramine	6.5 (6.6)	7.9 (7.6)	6.0 (6.1)	7.6 (6.9)	(6.4)
p-F-HHSiD	7.2 (7.3)	6.0 (6.6)	7.9 (7.5)	ND (7.2)	(6.7)
pirenzepine	8.3 (8.0)	6.8 (6.3)	6.9 (6.8)	7.7 (7.0)	(6.9)
tripitramine	ND (8.4)	9.7 (9.4)	6.5 (7.1)	ND (7.8)	(7.3)

The values in parentheses denote the affinity estimated in radioligand binding studies at recombinant human muscarinic receptors, conducted using [^{3}H]NMS as the radioligand in Tris-Krebs buffer (Bonhaus and Eglen, unpublished observations). The remaining values are affinities estimated functionally.

ND, not determined.

and Cobbin, 1986; Lazareno et al., 1990). Novel antagonists, such as tripitramine (M_2 selective; Melchiorre et al., 1993), darifenacin (M_3 selective; Wallis et al., 1995) or a compound isolated from a snake toxin, MT3 (Jolkkonen et al., 1994), may also prove to be important discriminative tools.

1. Classification issues. To implicate a muscarinic receptor subtype in a smooth muscle response relies upon operational characterization on the basis of ligand affinity; an approach not without its problems. Several discrepancies, for example, exist with regard to the affinities of antagonists at cloned and endogenous muscarinic receptors. These differences arise from variations in the cell transfection systems, the level of membrane receptor glycosylation or lipid concentration (see Richards, 1991 for a review of this area). Furthermore, irrespective of the origin of the muscarinic receptor (endogenous or recombinant), differences occur between affinity values estimated from radioligand binding studies and those obtained from functional studies. These disparities arise from the use of hypotonic buffers in binding studies and higher ionic strength buffers in pharmaco-

TABLE 3

Comparison of affinity estimates for zamifenacin, darifenacin and p-F-HHSiD in radioligand binding studies and functional studies

Receptor	Tissue/Cloned receptor	Zamifenacin	Darifenacin	p-F-HHSiD
M_1	Rabbit vas deferens	7.4[a]	7.8[f]	6.7[n]
	Canine saphenous vein	7.9[b]	8.1[g], 7.9[h]	7.1[k]
	Cloned human receptor	(7.5[c])	(7.5[i])	(7.7)[l]
M_2	Guinea pig atria	7.1[a], 6.6[b]	7.3[f], 6.9[h]	6.0[k]
	Cloned human receptor	(7.1[c])	(7.4[i])	(6.9)[l]
M_3	Guinea pig ileum	9.3[a], 9.3[b]	9.1[g], 9.4[h]	7.9[k]
	Guinea pig esophagus	8.8[b]	9.5[h]	8.2[k]
	Guinea pig trachea	8.1[a], 8.2[b]	8.7[g], 9.3[h]	7.1[k]
	Guinea pig bladder	7.6[b]	8.7[f]	7.6[k]
	Rat bladder	8.3[d]	8.3[d]	7.4[d]
	Human bladder	ND	8.7[o]	ND
	Dog ciliary muscle	<6[e]	ND	ND
	Dog ileum	8.6[e]	ND	ND
	Human bronchus	7.6[j]	ND	6.7[j]
	Human colon	ND	ND	6.8–7.4[m]
	Cloned human receptor	(7.9[c])	(8.4[i])	7.8[l]
M_4	Cloned human receptor	(6.7[c])	(7.9[i])	(7.5)[l]
m5	Cloned human receptor	ND	(7.9[i])	(7.0)[l]

[a] Wallis, 1995.
[b] Watson et al., 1995e.
[c] Eglen et al., (unpublished observations).
[d] Hegde et al., 1996.
[e] McIntyre and Quinn, 1995.
[f] Newgreen and Naylor, 1996a.
[g] Wallis et al., 1995.
[h] Eglen et al., 1996b.
[i] Nunn et al., 1996.
[j] Watson et al., 1995b.
[k] Eglen et al., 1990b.
[l] Dorje et al., 1991b.
[m] Kerr et al., 1995.
[n] Lambrecht et al., 1989a, b.
[o] Newgreen and Naylor, 1996b.
ND, not determined.
Values in parentheses denote binding affinities, all remaining values are functional affinity estimates.

logical experiments (Hulme et al., 1990). In binding studies using hypotonic buffers alter antagonist affinities at muscarinic M_2 receptors, influencing the selectivity of antagonists for muscarinic M_3 over M_2 receptors (Pedder et al., 1991; Hou et al., 1996).

This phenomena is illustrated by determination of the selectivity of the muscarinic M_3 antagonists, zamifenacin (Watson et al., 1995f) and darifenacin (Wallis et al., 1995; Nunn et al., 1996; Eglen et al., 1996b) (fig. 2). Thus, in radioligand binding studies using recombinant human muscarinic receptors, the muscarinic M_3 selectivity over M_2 receptors for both these compounds is less than that found in functional studies at endogenous muscarinic M_2 and M_3 receptors (table 3). In general, this effect results in an underestimation of the selectivity of novel antagonists and implies that functional studies be undertaken in parallel with binding studies to best define subtype selectivity. Even under ideal conditions, the selectivities of muscarinic antagonists between subtypes are low (table 2). Because of this limited selectivity of the antagonists available between muscarinic receptors, errors in affinity estimation complicate the operational characterization of the muscarinic subtype, and the importance of estimating ligand affinities under conditions of equilibrium cannot be overestimated.

The ability to selectively stimulate a muscarinic receptor subtype is not feasible at present, because subtype-selective agonists are unavailable (Caulfield, 1993). Muscarinic agonists, even those reported as selective, are correctly designated as 'functionally selective,' because their discrimination between subtypes depends upon the prevailing receptor reserve, ability to impart a conformational receptor change and/or the stimulus-response coupling efficiency, in addition to the nature of the muscarinic receptor subtype activated (Eglen and Watson, 1996; Wang and El-Fakahany, 1993; Heldman et al., 1996 for further discussion). This problem occurs when studying agonists in recombinant systems (Richards and van Giersbergen, 1995) but is also an issue in studies of smooth muscle function. At muscarinic receptors in dispersed cells from intestinal smooth muscle, the potencies of muscarinic agonists are very high, suggesting that removal of diffusional barriers alone increases agonist potency (Grider et al., 1987). The potency (pEC_{50}) of (+)*cis* dioxolane, a highly potent, yet non-selective agonist at muscarinic M_3 receptors mediating contraction, varies from 7.9 (guinea pig isolated trachea) to 6.0 (rabbit isolated aorta; Watson and Eglen, 1994b). Indeed, the purportedly muscarinic M_1 receptor selective agonist, McN-A-343 (Hammer and Giachetti, 1982), causes contractile responses in the guinea pig isolated taenia that are mediated by muscarinic M_3 receptors (Eglen et al., 1987). Moreover, McN-A-343 has been shown to act as a selective muscarinic M_4 agonist in recombinant systems (Richards and van Giersbergen,

TABLE 4

Affinity estimates of key ligands at tissues of the gastrointestinal tract in functional studies

Tissue	Species	Atrop	Piren	Meth	AF-DX	4-DA	*p*-F-H	Himb	Tripit	Reference
Ileum	Dog	9.3	7.0	6.9	–	8.8	–	–	–	McIntyre and Quinn, 1995[a]
	Rat	8.9	–	5.8	–	–	–	–	–	Melchiorre et al. 1987[a]
		–	–	6.5	6.5	–	–	8.1	–	Brunner, 1989[b]
		9.5	6.7	6.3	6.6	8.9	–	7.4	–	Lazareno and Roberts, 1989[a]
		–	–	–	–	–	7.9	–	–	Lambrecht et al., 1989a,b[a]
		–	6.9	5.6	6.0	–	7.5	–	–	Candell et al., 1990[a]
		–	–	–	–	–	–	–	6.5	Chiarini et al., 1995[a]
	Guinea pig	9.1	6.8	–	–	9.0	–	–	–	Clague et al. 1985[a]
		–	–	–	–	9.0	–	–	–	Barlow et al., 1980[a]
		8.7	6.5	6.1	–	8.8	–	–	–	Barocelli et al., 1994a[a]
		–	6.7	–	–	–	7.8	–	–	Bognar et al., 1992[a]
		9.1	–	–	–	–	–	7.3	–	Gilani and Cobbin, 1986[a]
		8.9	–	6.2	–	–	–	–	–	Melchiorre et al., 1987[a]
		9.1	6.8	–	5.7	9.0	–	–	–	Eglen et al., 1987a[a]
		–	6.9	6.2	–	–	7.8	–	–	Lambrecht et al., 1988[a]
		8.9	–	6.2	–	–	–	–	–	Waelbroeck et al., 1989[a]
		–	–	–	–	–	7.9	–	–	Eglen et al., 1990b[a]
		9.0	6.8	5.9	–	8.9	7.9	–	–	Eglen et al., 1992b[a]
		–	–	6.0	–	–	–	–	–	Ford et al., 1991[a]
		9.1	–	6.3	–	8.9	7.1	–	–	Ford et al., 1991[b]
		8.9	6.8	–	6.6	–	–	–	–	Dorofeeva et al., 1992[a]
		9.4	7.2	–	6.8	–	–	–	–	Wallis et al., 1993[a]
		10.3	6.5	6.1	–	8.3	7.3	–	–	Barocelli et al., 1994b[a]
		–	–	–	–	–	–	–	6.5	Chiarini et al., 1995[a]
		9.2	6.4	–	5.6	–	–	–	–	Doods et al., 1994
		9.6	6.9	–	6.6	9.4	–	–	–	Kurtel et al., 1990[a]
Esophagus	Guinea pig	9.2	7.4	6.0	6.3	9.0	–	–	–	Eglen and Whiting, 1988[a]
	Rat	9.1	6.8	6.2	–	8.7	7.5	7.2	–	Eglen et al., 1996a[a]
	Dog	9.1	7.1	–	6.7	9.0	–	–	–	Lad et al., 1991[c]
Gastric fundus	Guinea pig	8.2	7.1	–	5.7	–	–	–	–	Del Tacca et al., 1990[a]
		9.0	7.0	6.0	–	9.6	8.1	7.2	–	Eglen et al., 1992b[a]
Gall bladder	Guinea pig	–	–	7.7	–	–	7.6	–	–	Ozkutlu et al., 1993[a]
		8.4	7.9	–	6.7	8.3	–	–	–	Kurtel et al., 1990[a]
Bile duct	Guinea pig	9.6	7.3	–	6.9	9.0	–	–	–	Karahan et al., 1991[a]
Colon (circular)	Human	8.7	7.2	–	7.4	9.4	6.9	7.5	–	Kerr et al., 1995[a]
Colon (longitudinal)	Human	8.6	6.9	–	6.4	9.1	7.4	7.5	–	Kerr et al., 1995[a]
Taenia caeci	Guinea pig	8.7	6.3	–	–	8.7	–	–	–	Eglen et al., 1987[a]
Anococcygeus	Rat	9.1	–	–	–	8.8	–	–	–	Oriowo, 1982[a]
Rectum	Rat	9.0	7.2	–	–	8.2	–	–	–	Akah and Oriowo, 1985[a]

[a] Denotes affinity estimates obtained against contraction.

[b] Denotes affinity estimates obtained against phosphoinositide hydrolysis and/or adenylyl cyclase inhibition.

[c] Denotes affinity estimates obtained against short-circuit current.

Atrop, atropine; Piren, pirenzepine; Meth, methoctramine; AF-DX, AF-DX 116; 4-DA, 4-DAMP; *p*-F-H, *p*-F-HHSiD; Himb, himbacine; Tripit, tripitramine.

1995), but is inactive at muscarinic M_1 receptors mediating contraction of canine saphenous vein (Watson et al., 1995e). The selectivity, therefore, of McN-A-343 for muscarinic M_1 receptors appears not to rest upon a differential affinity for this subtype (Micheletti and Schiavone, 1990). Care should thus be taken in the use of agonists to characterize muscarinic receptor subtypes in smooth muscle, since their potency capriciously depends upon the receptor reserve, response measured and developmental state of the muscle, in addition to the muscarinic receptor subtype activated (Grana et al., 1987; Ford et al., 1991; Zhang, 1996). These problems are exacerbated when purportedly selective agonists are used in vivo to elucidate the role of muscarinic receptor subtypes (e.g., Williams et al., 1992).

Taken together, to best implicate a muscarinic receptor in mediation of a smooth muscle response, a profile of antagonist affinities should be determined by functional methods using the null hypothesis (Eglen and Whiting, 1986; Caulfield, 1993; Eglen and Watson, 1996). This point is critical in the classification of muscarinic receptors, in general, and smooth muscle, in particular, because no antagonist is preferential for one subtype over the remaining four (Hulme et al., 1990; Caulfield, 1993) (table 2). A summary of antagonist affinities is shown in tables 4-7. As discussed above, the utility of determining such a profile in muscarinic receptor classification presumes equilibrium conditions and involvement of a single muscarinic receptor subtype in the response. This presumption is unlikely when studying smooth muscle,

TABLE 5

Affinity estimates of key ligands at tissues of the respiratory tract in functional studies

Tissue	Species	Atrop	Piren	Meth	AF-DX	4-DA	*p*-F-H	Himb	Tripit	Reference[a]
Trachea	Cow	9.0	6.9	6.5	6.3	9.0	–	–	–	Roffel et al., 1988
		–	6.9	–	6.3	8.5	–	–	–	Roffel et al., 1989
	Horse	–	6.8	–	–	8.5	–	–	–	Yu et al., 1992
	Guinea pig	9.2	–	–	–	–	–	7.1	–	Gilani and Cobbin, 1986
		9.1	7.1	–	–	9.1	–	–	–	Eglen et al., 1987
		9.1	6.9	6.1	6.2	9.1	–	–	–	Eglen and Whiting, 1988
		–	–	6.1	–	–	–	7.6	–	Eglen et al., 1988
		–	–	5.4	–	–	–	–	–	Giraldo et al., 1988
		8.8	6.9	6.2	–	8.8	7.3	–	–	Eglen et al., 1990
		–	–	6.0	–	8.7	7.0	–	–	Eglen et al., 1991
		9.0	6.9	–	6.2	–	–	–	–	Dorofeeva et al., 1992
		9.4	6.5	5.5	–	–	7.2	–	–	Watson and Eglen, 1994a
		9.2	7.2	–	6.9	–	–	–	–	Wallis et al., 1993
		–	–	6.3	–	–	–	–	6.3	Chiarini et al., 1995
	Mouse	8.6	6.5	–	6.3	8.7	–	–	–	Garssen et al., 1993
	Rabbit	8.4	6.8	–	6.5	9.1	–	–	–	Mahesh et al., 1992
		9.0	7.1	6.1	6.4	–	7.4	–	–	Eltze and Galvan, 1994
	Rat	9.6	7.0	6.6	6.4	9.1	7.6	–	–	Kirkup and Moore, 1995
Bronchi	Dog	8.5	7.0	–	6.8	8.6	–	–	–	Itabashi et al., 1991
	Human	–	6.8	5.5	–	9.0	–	–	–	Roffel et al., 1989
		9.1	6.8	5.3	–	9.4	6.7	7.0	–	Watson et al., 1995a
Lung	Guinea pig	–	6.8	6.1	–	8.5	–	–	–	Haddad et al., 1991
			6.4	7.3	6.6	–	–	–	–	Roffel et al., 1993a
				7.0	–	–	–	–	7.9	Chiarini et al., 1995
	Rat	9.0	7.4	–	5.9	9.4	–	–	–	Post et al., 1991

[a] For all references: denotes affinity estimates obtained against contraction.

Atrop, atropine; Piren, pirenzepine; Meth, methoctramine; AF-DX, AF-DX 116; 4-DA, 4-DAMP; *p*-F-H, *p*-F-HHSiD; Himb, himbacine; Tripit, tripitramine.

since many smooth muscles express multiple muscarinic receptors (see Eglen et al., 1994b for a recent review) (table 8). The antagonist affinity value derived, therefore, may be a resultant value, arising from involvement of more than one muscarinic receptor subtype in the response. Reassuringly, in most smooth muscles, one muscarinic receptor subtype participates in the response (tables 4-7), unless specialized experimental conditions prevail e.g., muscarinic M_3 receptor depletion and concomitant elevation of adenylyl cyclase activity (Thomas et al., 1993; Eglen et al., 1994a).

A final issue is that functional analysis of muscarinic receptors in smooth muscles is not, in itself, sufficient to fully assess their role in muscle function, because the approach yields no information as to the receptor density or, indeed, the proportions of different muscarinic receptor subtypes expressed (Brann et al., 1993). Consequently, radioligand binding studies, reverse transcript-polymerase chain reaction (RT-PCR) studies (Kajimura et al., 1992), northern blot techniques (to determine the messenger ribonucleic acid (mRNA) species present; Maeda et al., 1988) and immunoprecipitation experiments (to estimate the receptor protein species expressed; Wall et al., 1991; Levey, 1993; Yasuda et al., 1993) should be used in concert with operational approaches to provide full characterization.

2. Muscarinic M_1 receptors. Muscarinic M_1 receptors exhibit a high affinity toward pirenzepine and 4-DAMP, an intermediate affinity for *p*-F-HHSiD and a low affinity for methoctramine, darifenacin or himbacine. Pirenzepine is well established as a selective muscarinic M_1 receptor antagonist, although the selectivity between muscarinic M_1 and M_4 receptors is small (table 2). Analogues of pirenzepine, such as the isomers of telenzepine, also have a similar selectivity profile (Schudt et al., 1989). Other muscarinic M_1 antagonists, as yet not extensively characterized, include caramiphen and the iodo or nitro analogues (Hudkins et al., 1993) and S-(−)-α-(hydroxymethyl)benzeneacetic acid 1-methyl-4-piperdinyl ester (S-(−)-ET 126; Ghelardini et al., 1996). The real selectivity of these ligands is not as yet clear because the affinity data at muscarinic M_4 receptors are presently unavailable.

3. Muscarinic M_2 receptors. The muscarinic M_2 receptor exhibits high affinity toward AF-DX 116, methoctramine and himbacine, but a low affinity for pirenzepine, 4-DAMP, darifenacin and *p*-F-HHSiD (table 2). Structural alterations have been made to pirenzepine, a prototypic muscarinic M_1 receptor antagonist (Jaen and Davis, 1994). AF-DX 116 is an example of one such compound (Hammer et al., 1986); because of its selectivity for M_2 and M_4 muscarinic receptors, it has been used extensively in receptor classification. An analogue of hexamethonium, heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl ammonium bromide) is also selective toward the muscarinic M_2 receptor (Choo and Mitchelson, 1989). Some analogues of AF-DX 116, including AQ-RA

TABLE 6

Affinity estimates of key ligands at tissues of the genitourinary tract in functional studies

Tissue	Species	Atrop	Piren	Meth	AF-DX	4-DA	*p*-F-H	Himb	Tripit	Reference
Uterus	Guinea pig	–	6.6	7.9	7.1	–	–	–	–	Eglen et al., 1989[b]
		9.1	7.0	–	9.0,7.0	9.6	–	–	–	Lieber et al., 1990[a]
		8.9	7.0	–	6.5	9.5	–	–	–	Leiber et al., 1990[b]
		9.4	6.5	–	8.9	6.5	–	–	–	Leiber et al., 1990[c]
		–	7.0	7.5	–	8.9	–	7.9	–	Dorje et al., 1990[a]
		8.9	6.8	6.8	–	8.9	–	7.7	–	Eglen et al., 1992a[a]
		9.2	6.8	–	6.4	–	–	–	–	Dorofeeva et al., 1992[a]
		–	6.6	–	–	–	6.3	–	–	Bognar et al., 1992[a]
		8.7	6.6	7.2	6.9	8.3	6.8	7.9	–	Doods et al., 1993[a]
Urinary bladder	Guinea pig	8.6	6.6	–	6.4	–	–	–	–	Noronha-Blob et al., 1989[a]
		8.6	6.8	–	6.4	–	–	–	–	Del Tacca et al., 1990[a]
		–	–	–	–	–	7.6	–	–	Eglen et al., 1990b[a]
		8.9	6.7	–	6.2	–	–	–	–	Dorofeeva et al., 1992[a]
	Human	9.1	6.9	–	–	–	–	–	–	Poli et al., 1992[a]
		9.4	6.9	6.3	–	9.2	7.4	–	–	Harriss et al., 1995[b]
		9.3	6.6	5.3	–	–	–	–	–	Newgreen and Naylor, 1996b[a]
	Mouse	8.9	6.8	–	–	–	–	–	–	Durant et al., 1991[a]
	Rabbit	9.3	–	–	–	–	–	–	–	Downie et al., 1977[a]
		–	–	6.7	5.5	9.2	7.1	–	–	Wang et al., 1995[a]
		–	7.1	6.7	–	9.1	–	–	–	Tobin and Sjogren, 1995[a]
	Rat	9.4	6.8	5.8	6.2	9.2	–	–	–	D'Agostino et al., 1993[a]
		9.3	7.1	6.8	–	9.0	7.3	–	–	Longhurst et al., 1995[a]
		–	–	6.1	–	10.6	7.8	–	–	Wang et al., 1995[a]
		9.1	6.8	5.9	–	8.9	7.4	–	–	Hegde et al., 1996[a]
Ureter	Pig (tonic)	10.8	8.6	8.1	6.9	9.4	8.5	–	–	Hernandez et al., 1993[a]
	(phasic)	10.6	7.9	8.4	7.8	9.6	8.3	–	–	Hernandez et al., 1993[a]
Vas deferens	Human	8.8	7.4	–	5.9	–	–	–	–	Miranda et al., 1992[a]
	Rat	9.1	–	–	–	–	–	–	–	Doggrell, 1986[a]
		8.5	8.1	–	–	9.1	8.5	–	–	Miranda et al., 1994[a]

[a] Denotes affinity estimates obtained against contraction.

[b] Denotes affinity estimates obtained against phosphoinositide hydrolysis.

[c] Denotes affinity estimates obtained against adenylyl cyclase inhibition.

Abbreviations; Atrop, atropine; Piren, pirenzepine; Meth, methoctramine; AF-DX, AF-DX 116; 4-DA, 4-DAMP; *p*-F-H, *p*-F-HHSiD; Himb, himbacine; Tripit, tripitramine.

741 (Doods et al., 1991) have improved muscarinic M_2 receptor selectivity, although these have not been as extensively studied. Guanylpirenzepine, at least in radioligand binding studies, exhibits some degree of selectivity for recombinant muscarinic M_2 receptor, over the muscarinic M_1, M_3 and M_4 receptors (Lazareno et al., 1990). Other putative muscarinic M_2 antagonists include several cervane alkaloids, such as imperialine (Eglen et al., 1992b) and the chlorinated derivative (Baumgold et al., 1994).

One problem associated with these latter compounds is the limited discrimination between muscarinic M_2 and M_4 receptors (Caulfield, 1993). (S) dimethindene, although possessing histamine H_1 receptor antagonist activity, is selective for muscarinic M_2 receptors over M_1, M_3 or M_4 receptors (Pfaff et al., 1995). One analogue of methoctramine, tripitramine (Melchiorre et al., 1993; Maggio et al., 1994; Angeli et al., 1995; Chiarini et al., 1995), is several-fold more selective for muscarinic M_2 over the M_4 receptor in comparison with the other 'selective' M_2 antagonists discussed. However, this separation is less evident at human recombinant muscarinic M_1 and M_2 receptors than at endogenously expressed receptors (Chiarini et al., 1995). Several compounds, believed to be selective for muscarinic M_2 receptors, including gallamine (Hulme et al., 1990), bis-quaternary heptane-1,7-bis(dimethyl-3′-phthalimidopropyl) ammonium (Christopoulos and Mitchelson, 1994), methoctramine (Eglen et al., 1988) and himbacine (Lee and El-Fakahany, 1990), allosterically modulate muscarinic receptor function. Such allosterism, most pronounced at M_2 receptors, complicates the determination of their affinity and the subsequent interpretation of their selectivity (Proska and Tucek, 1995; see Tucek and Proska, 1995, for review). This problem is germane to studies of the function of smooth muscle, where muscarinic M_2 and M_3 receptors are co-expressed and their pharmacological discrimination is critical.

4. Muscarinic M_3 receptors. The muscarinic M_3 receptor exhibits a high affinity for 4-DAMP (Barlow et al.,

TABLE 7
Affinity estimates of key ligands at vascular and ocular smooth muscles in functional studies

Tissue	Species	Atrop	Piren	Meth	AF-DX	4-DA	*p*-F-H	Himb	Tripit	Reference
Coronary artery	Bovine	–	6.9	–	6.3	–	–	–	–	Duckles, 1988[a]
	Pig	9.5	7.3	5.6	6.2	9.1	–	–	–	Van Charldorp and Van Zwieten, 1989[a]
Basilar artery	Pig	9.2	6.3	5.6	7.5	–	–	–	–	Van Charldorp and Van Zwieten, 1989[a]
Pulmonary artery	Human	9.4	6.7	5.4	–	–	7.4	–	–	Norel et al., 1996[a]
	Rat	–	7.0	5.5	–	9.2	–	–	–	McCormack et al., 1988[a]
Femoral artery	Cat	9.7	7.2	–	6.0	9.6	–	–	–	Fernandes et al., 1991[a]
Aorta	Rabbit	8.1	–	6.7	7.1	–	–	–	–	Jaiswal et al., 1991[a]
		9.4	6.6	5.9	–	9.2	7.7	7.1	–	Watson and Eglen, 1994b[a]
	Rat	9.5	6.7	6.3	–	9.4	–	–	–	Boulanger et al., 1994[a]
		9.2	–	–	–	9.6	–	–	–	Boulanger et al., 1994[a]
Coronary artery	Horse	10.1	7.6	5.8	–	9.8	7.3	–	–	Obi et al., 1994[a]
Uterine artery	Guinea pig	9.6	6.7	6.1	–	–	7.8	–	–	Jovanovic et al., 1994[a]
Perfused kidney	Rat	8.4	6.2	–	–	8.3	6.0	5.9	–	Eltze et al., 1993[a]
Saphenous vein	Dog	8.9	8.1	–	–	–	–	–	–	O'Rourke and Vanhoutte, 1987[a]
		–	8.1	6.2	–	–	7.0	–	–	Eglen et al., 1990[a]
		–	8.1	6.2	–	8.4	7.2	7.3	–	Watson et al., 1995e[a]
Femoral vein	Dog	–	8.0	6.3	–	–	6.9	–	–	Eglen et al., 1990a[a]
Iris	Rabbit	–	7.3	–	–	–	6.4	–	–	Bognar et al., 1992[a]
		–	6.9	5.9	–	9.1	–	6.4	–	Fuder et al., 1989[a]
	Rat	–	7.2	6.4	6.7	9.0	–	–	–	Masuda et al., 1995[b]
		–	7.2	<5.0	6.5	8.9	–	–	–	Masuda et al., 1995[a]
Ciliary muscle	Human	9.1	6.8	–	–	9.3	7.8	–	–	Matsumoto et al., 1994[c]

[a] Denotes affinity estimates obtained against contraction.
[b] Denotes affinity estimates obtained against relaxation.
[c] Denotes affinity estimates obtained against phosphoinositide hydrolysis.
Atrop, atropine; Piren, pirenzepine; Meth, methoctramine; AF-DX, AF-DX 116; 4-DA, 4-DAMP; *p*-F-H, *p*-F-HHSiD; Himb, himbacine; Tripit, tripitramine.

1980) and darifenacin a moderate affinity for *p*-F-HHSiD, but a low affinity for pirenzepine, methoctramine and himbacine (table 3). Analogues of 4-DAMP with better muscarinic M_3 over M_2 receptor selectivity include pentamethylene bis-4-DAMP (Barlow and Shepherd, 1985), 4-DAMP mustard (Barlow et al., 1990) and benzyl-4-DAPine (Barlow et al., 1992). Subsequent studies (Caulfield et al., 1993) have not confirmed the selectivity of the benzyl-4-DAPine, possibly due to problems of solubility (Barlow et al., 1995). The 3-chloro derivative of 4-DAMP, N-(3-hydroxypropyl)-4-piperidinyl diphenylacetate (3-CP-4-DAP) forms a stable azetidium ion in aqueous solution that acts as a reversible, high affinity ligand for muscarinic M_1, M_3, M_4 and m5 receptors, with a 10-14-fold lower affinity for muscarinic M_2 receptors (Ehlert et al., 1996). N-2-chloroethyl-4-piperidinyl diphenyl acetate (4-DAMP mustard) has been used to alkylate muscarinic M_3 receptors (Barlow et al., 1990; 1991; Griffin et al., 1993) in smooth muscle. Although the selectivity of the compound between muscarinic receptor subtypes is low (Waelbroeck et al., 1992; Eglen and Harris, 1993a), selective muscarinic M_3 receptor alkylation can be enhanced in the presence of a reversible M_2 receptor antagonist, such as AF-DX 116 or methoctramine, as a protecting agent (Thomas et al., 1993; see Eglen et al., 1994a for review).

Nonetheless, there are emerging functional data to suggest that muscarinic M_3 receptors are pharmacologically different. Muscarinic M_3 receptors in rat ileum may differ from those in rat urinary bladder because of differences in potency of isomers of agonists structurally related to (+)*cis*-dioxolane (Angeli et al., 1988). Some muscarinic M_3 receptor antagonists also distinguish between various muscarinic M_3 receptors, even in smooth muscle from the same species (Eglen et al., 1990b; Dorofeeva et al., 1992). Zamifenacin (Wallis, 1995; fig. 2), for example, is structurally related to benzyl-4-DAPine (Barlow et al., 1992) and displays higher affinities for smooth muscle muscarinic M_3 receptors in guinea pig ileum and esophagus in comparison with muscarinic M_3 receptors in trachea and bladder (Wallis et al., 1993; Watson et al., 1995f) (table 3). Selectivity is also seen with this compound in vivo, because, in dogs, inhibition of gastrointestinal motility occurs at doses that do not effect pupil diameter (McRitchie et al., 1993). This finding is supported by in vitro data indicating that zamifenacin discriminates, by more than two orders of magnitude, between muscarinic M_3 receptors in canine ciliary muscle and ileum (McIntyre and Quinn, 1995). In similar assays, a related compound, darifenacin, also distinguishes between muscarinic M_3 receptors, albeit to a lesser degree (Wallis et al., 1995; Eglen et al., 1996b). Both zamifenacin and darifenacin functionally discriminate between muscarinic M_3 receptors in canine salivary gland and ileum (Wallis et al., 1993; Wallis, 1995; Sawyer et al., 1996). It is unknown, however, whether

TABLE 8

Smooth muscle muscarinic receptor heterogeneity identified in radioligand binding studies

Tissue	Species	Pirenzepine M_2:M_3	AF-DX 116 M_2:M_3	Methoc. M_2:M_3	Himbacine M_2:M_3	4-DAMP M_2:M_3	HHSiD M_2:M_3	Reference
Ileum	Guinea pig	–	65%35% (7.0:5.8)	–	–	–	–	Michel and Whiting, 1987
		–	82%:18% (6.9:5.6)	–	–	–	–	Giraldo et al., 1988
		–	65%35% (7.2:5.7)	–	–	–	–	Giraldo et al., 1988
		–	77%23% (7.0:6.0)	80%:20% 9.2:7.7	–	–	100% 7.4	Michel and Whiting, 1988
		–	–	70%:30% (7.7):(6.2)	–	100% (8.4)	–	Ford et al., 1991
	Rat	–	70%:30% (7.5):(6.4)	70%:30% (8.4):(6.8)	70%:30% (8.6):(7.3)	70%:30% (8.1):(8.8)	–	Lazareno and Roberts, 1989
Stomach	Human	–	79%:21% (6.5):(5.5)	–	–	–	–	Bellido et al., 1995
Taenia caeci	Guinea pig	–	70%:30% (7.8):(6.6)	–	–	–	–	Elnatan and Mitchelson, 1993
Colon	Dog	82%:18% (5.4:7.6)	–	–	–	–	–	Zhang et al., 1991
	Human	–	76%24% (6.3:5.1)					Gomez et al., 1992
	Rat	–	39%:61% (5.2:6.5)	–	–	–	–	Gomez et al., 1992
	Rat (adult)	–	100% (6.5)	–	–	49%:51% (7.3:8.6)	–	Zhang, 1996
	Rat (neonate)	–	100% (6.7)	–	–	100% (7.5)	–	Zhang, 1996
Trachea	Cow	–	74%:26% (7.4):(5.6)	83%:17% (7.8):(5.4)	–	100% (8.0)	100% (6.8)	Roffel et al., 1988
		–	85%:15% (6.9):(5.6)	–	–	15%:85% (ND): (8.2)	–	Lucchesi et al., 1990
	Calf	–				40%:60%		Roets et al., 1992
	Dog	–	–	72%:28% (7.6):(5.3)	–	45%:55% (7.3):(8.7)	44%/56% (6.7):(7.6)	Yang, 1991
		–	89%:11% (7.1):(4.8)	–	–	–	100%	Fernandes et al., 1992
	Guinea pig	–	52%:48% (6.9):(5.5)	64%:36% (7.5):(5.5)	–	100% (7.8)	–	Haddad et al., 1991
	Rabbit	–	83%:17% (8.0):(6.7)	–	–	76%:24% (8.8):(10.4)	72%:28% (6.6):(8.0)	Mahesh et al., 1992
Trachea and bronchi	Pig	–	–	70%:30% (8.4):(6.6)	–	100% (8.4)	–	Haddad et al., 1994
Bladder	Rat	–	87%:13% (6.9):(5.4)	–	–	–	–	Monferini et al., 1988

Values in parentheses correspond to the pK_i values at the different receptor populations.
ND, not determined; Methoc., methoctramine.

the structurally related compound, benzyl-4-DAPine (Barlow et al., 1992) exhibits similar differences in muscarinic M_3 receptor affinities. The muscarinic M_3 receptor antagonist, *p*-F-HHSiD discriminates, by about 10-fold, between smooth muscle M_3 receptors in guinea pig (Eglen et al., 1990a, b; Roffel et al., 1994b) tracheal and ileal smooth muscle (table 3). However, in contrast to zamifenacin (Watson et al., 1995e), *p*-F-HHSiD does not discriminate between ileum and urinary bladder muscarinic M_3 receptors (Eglen et al., 1990b). Relatively low affinities for this antagonist have also been reported at muscarinic M_3 receptors mediating contraction of human colonic circular muscle (Kerr et al., 1995) or human bronchus (Watson et al., 1995a). A summary of these differing affinity values for these antagonists is given in table 3. Some of these differences between muscarinic

M_3 receptors (approximately three orders of magnitude, in the case of zamifenacin) are greater than some differences in affinities between other muscarinic receptor subtypes (table 2).

It may be premature to postulate different muscarinic M_3 receptor subtypes, given the identity in cloned muscarinic m3 sequences from different tissues and thus the identification of one, intronless, muscarinic m3 receptor gene. Other potential factors that would affect ligand affinity, such as the membrane lipid composition, the state of glycosylation, palmitolyation of the receptor (Richards, 1991), as well as the degree of pre-coupling, must be eliminated before postulating muscarinic M_3 receptor heterogeneity (Eglen et al., 1990b; Richards, 1991; Roffel et al., 1994b). Moreover, a characteristic of some G protein coupled receptors is that they are constitutively active i.e., they couple to a G protein in the absence of agonist. This phenomena may hold true for muscarinic receptor subtypes, both recombinant and endogenously expressed (Jakubik et al., 1995). Muscarinic receptor antagonists, such as atropine, have a higher affinity for, and thus stabilize, the inactive conformation of the receptor and act as inverse agonists (Jakubik et al., 1995). It is not known whether this phenomena occurs with muscarinic receptors in smooth muscle.

5. Muscarinic M_4 and m5 receptors. The muscarinic M_4 receptor remains difficult to define because methoctramine, and several other purportedly selective M_2 receptor antagonists, exhibit high affinity for this subtype. The concurrent affinity of himbacine and *p*-F-HHSiD, however, can serve to distinguish the receptor from muscarinic M_1 and M_2 subtypes, respectively (table 2). Radioligand binding studies also have suggested that dicyclomine, DAU 5884, DAU 6202 and AQ-RA 721 distinguish muscarinic M_4 from M_2 receptors (Doods et al., 1993). Tripitramine (Melchiorre et al., 1993) could also prove useful to distinguish between those responses mediated by muscarinic M_2 and those by M_4 receptors, the advantage of this compound being the low affinities for other muscarinic receptor subtypes. MT3, isolated from venom of *Drendroaspis angusticeps* exhibits high affinities at muscarinic M_4 and M_1 receptors, with little binding detectable at muscarinic M_2 and M_3 receptors (Jolkkonen et al., 1994). If confirmed, this ligand (MT3) will provide the most selective muscarinic receptor antagonists identified to date.

The pharmacology of the m5 gene product differs from that of other muscarinic receptors (Hulme et al., 1990), although no single ligand is preferential toward the expressed protein. In transfected Sf9 insect cells, promethazine and pilocarpine are marginally (4–7-fold) selective toward this receptor over the remaining four (Dong et al., 1995). It remains to be established whether this holds true in a mammalian expression system. Collectively, however, the lack of selective antagonists and restricted distribution of this receptor account for the limited knowledge regarding its physiological role.

III. Muscarinic Receptors and Smooth Muscle

In hollow organs, including the alimentary tract and genitourinary system, smooth muscle is comprised of an outer longitudinal layer and an inner circular muscle layer. Smooth muscle cells vary between 30-450 nm in length and 2-6 μm in diameter. These myocytes are surrounded by the basal lamina, and organized into bundles, separated by an extracellular space, approximately 100 nm wide. The extracellular space is filled with nerves, among other cells. Most smooth muscles are innervated by post-ganglionic nerve fibers of the autonomic nervous system. In many tissues, the cell bodies of post-ganglionic fibers of the parasympathetic nervous system, specifically, lie within the walls of the tissues innervated (fig. 1). A comprehensive review of smooth muscle anatomy can be found elsewhere (Burnstock, 1970; Brock and Cunnane, 1992).

The following sections discuss the role of muscarinic receptor subtypes in various smooth muscles. The order in which they are addressed generally reflects the extent of the literature reported with each tissue in this respect.

A. Gastrointestinal Smooth Muscle

The excitatory actions (motility and secretion) of acetylcholine on the alimentary tract are well established. By contrast, one report (Williams et al., 1992) has speculated that muscarinic agonists at very low concentrations exert a relaxant action in guinea pig, but this has not been confirmed (Gathers et al., 1993). Cholinergic nerves make synaptic contact with smooth muscle cells in this tissue where, it is presumed, muscarinic receptors principally reside (see Buckley and Caulfield, 1992 for review). Muscarinic receptors are present in smooth muscle from myenteric plexus, longitudinal and circular muscle, esophagus and colon (Morisset et al., 1981; Buckley and Burnstock, 1986). Furthermore, the release of acetylcholine can also be induced by distension of the gut and consequent activation of stretch receptors. This local reflex causes excitation of cholinergic interneurons and thus modulates the direction and magnitude of peristaltic activity (see Johnson et al., 1996 for further discussion). Muscarinic receptors also influence secretory activity in the alimentary tract, including the output of acid from the stomach (Hirschowitz et al., 1995).

1. Small intestine. Guinea pig and rat isolated ileum have been extensively used as models for the function of muscarinic receptors in smooth muscle (e.g., Barlow et al., 1972; 1976; Ford et al., 1991; Eglen et al., 1992a, b, c; Honda et al., 1993). The majority of studies have concentrated on the role of the receptor in contraction, although two studies have shown that muscarinic M_3 receptors increase electrolyte secretion (Carey et al., 1987; Kachur et al., 1990). Muscarinic M_1 receptors are localized to the myenteric plexus and are not expected to be present post-junctionally in ileal smooth muscle

(Buckley and Burnstock, 1986). Although muscarinic M_3 receptors mediate ileal contraction, a role for muscarinic M_2 receptors, in terms of modulation of cAMP driven relaxation, is evident under specialized experimental conditions (see Ehlert and Thomas, 1995; Eglen et al., 1994a, b for reviews). Selective muscarinic receptor alkylation (Eglen and Harris, 1993a) or desensitization (Eglen et al., 1992c) experiments have shown that muscarinic M_3 receptors, exclusively, mediate contraction under standard conditions. Operationally, the affinities for 4-DAMP (Barlow et al., 1972, 1976; Clague et al., 1985), pentamethylene bis-4-DAMP (Barlow and Shepherd, 1985), hexahydrosiladifenidol (Waelbroeck et al., 1989; Lambrecht et al., 1989 a, b), *p*-F-HHSiD (Lambrecht et al., 1988), UH-AH 37 (Doods and Mayer, 1989), isomers of 2-phenylcyclohexyl diethylaminoether (Lu et al., 1991), AQ-RA 391, AQ-RA 618 (Doods et al., 1993), DF 545 (Barocelli et al., 1994b), zamifenacin and darifenacin (Wallis, 1995; Wallis et al., 1995; Eglen et al., 1996b) in guinea pig or rat ileum are consistent with activation of muscarinic M_3 receptors. This also holds true for the low affinities of muscarinic M_2-selective compounds, including gallamine (Riker and Wescoe, 1951; Clark and Mitchelson, 1976), stercuronium (Li and Mitchelson, 1980), pancuronium (Leung and Mitchelson, 1982), TL-68 (Sahin and Ilhan, 1987), AF-DX 116 (Hammer and Giachetti, 1982), methoctramine (Melchiorre et al., 1987), imperialine (Eglen et al., 1992b) and tripitramine (Chiarini et al., 1995). In addition to whole ileum, muscarinic M_3 receptors mediate contraction of both ileal longitudinal muscle (Lazareno and Roberts, 1989; Eglen et al., 1992c) and circular smooth muscle (Doods et al., 1994; Dietrich and Kilbinger, 1995).

Given the extensive evidence for a role of muscarinic M_3 receptors in ileal contraction, it was initially surprising that radioligand binding studies only demonstrated a high preponderance of muscarinic M_2 receptors (Choo and Mitchelson, 1986; Michel and Whiting, 1987). However, in ileal circular muscle, inhibition studies using AF-DX 116 and dicyclomine identified two populations of sites, only one of which exhibited a pharmacology consistent with muscarinic M_3 receptors (Michel and Whiting, 1987). Giraldo et al. (1987, 1988), using AF-DX 116, further identified muscarinic M_2 and M_3 receptors in both longitudinal and circular muscle of guinea pig ileum. It was suggested that these data were consistent with the presence of a large muscarinic M_2 receptor population and a minor M_3 receptor population, through which the contraction was mediated. These findings were not confirmed by other workers using AF-DX 116 (Nilvebrant and Sparf, 1988) or pancuronium (Choo and Mitchelson, 1986), highlighting the difficulties of detecting muscarinic M_2 and M_3 receptors using hypotonic buffers with antagonists of low muscarinic M_2: M_3 selectivity (Michel and Whiting, 1990). Subsequently, muscarinic M_2 and M_3 receptors were unambiguously identified, in similar proportions to those identified with AF-DX 116, with more selective muscarinic M_2 antagonists, including methoctramine (Michel and Whiting, 1988; Eglen et al., 1992c; Ford et al., 1991) and heptane-1,7-bis(dimethyl-3-phthalimidopropyl ammonium bromide (Choo and Mitchelson, 1989). The minor muscarinic M_3 population in guinea pig ileal longitudinal muscle can be selectively labeled with the muscarinic M_3 ligand, [^{3}H]4-DAMP (Michel and Whiting, 1990). Nonetheless, some groups, using selective muscarinic M_3 receptor antagonists, UH-AH 37 (Doods and Mayer, 1989) or *p*-F-HHSiD (Michel and Whiting, 1990), have failed to identify muscarinic M_3 receptors. Although the reasons for this remain unclear, the relatively low selectivity of the ligands and the use of buffers of low ionic strength may cause problems. Indeed, muscarinic M_2: M_3 receptor heterogeneity identified by methoctramine (Michel and Whiting, 1988; Ford et al., 1991; Eglen et al., 1992c) in guinea pig ileum is not readily apparent using a Tris buffer, in contrast to use of Krebs buffer (Michel and Whiting, 1988; 1990).

Small intestine tissue from other species have been less intensively studied, although muscarinic M_3 receptors mediate contraction of rat ileum (Brown et al., 1980; Nedoma et al., 1985). Tien et al. (1985) suggested that muscarinic binding sites on rat ileal smooth muscle resembled those in atria, because of their low affinity for pirenzepine. As in guinea pig ileum, muscarinic M_2 receptors form the major population in rat ileal and duodenal smooth muscle. These receptors have been directly labeled using [^{3}H]AF-DX 384 (Entzeroth and Mayer, 1991) and defined in inhibition studies using [^{3}H]NMS (Brunner, 1989; Leibmann et al., 1992), respectively.

A predominant muscarinic M_2 receptor population in small intestine has been confirmed using other techniques. Northern blot studies in guinea pig and rat ileal tissue also suggest a high proportion of muscarinic M_2 receptors and a low proportion of M_3 receptors (Maeda et al., 1988; Ford et al., 1991). Immunoprecipitation studies in rat and rabbit tissue confirm the presence of muscarinic M_2 receptors (Wall et al., 1991; Dorje et al., 1991a), although the proportion of muscarinic M_1 receptors is higher and the expression of muscarinic M_3 receptors lower than that predicted from binding studies. These differences may be attributable to contamination from muscarinic M_1 receptors in the myenteric plexus or selective solubilization of receptor subtypes.

Surprisingly little work has been undertaken regarding the role of muscarinic receptor subtypes in the function of the small intestine in vivo. Osinski et al. (1994) showed that denervation, although not changing the contractile potency of agonists, affects the affinity of methoctramine. Additional experiments are required to assess whether this reflects disclosure of additional muscarinic receptor subtypes. Furthermore, little is known concerning adaptive changes in muscarinic receptor subtypes in the small intestine, although in rat

ileum, it appears as though the total population decreases as a function of age (Michalek et al., 1993).

2. Colon. Biochemical and northern blot studies have shown that both muscarinic M_2 and M_3 receptors are expressed in canine (Zhang et al., 1991; Zhang and Buxton, 1991) and mature rat colonic muscle (Zhang, 1996). Northern blot analysis of colon suggests m2 and m3 receptor mRNA expression, although the m3 probe hybridized to both 5.4 and 4.5 kb transcripts, the significance of which is unknown (Zhang et al., 1991). Muscarinic M_2 receptors mediate inhibition of adenylyl cyclase activity, via a pertussis toxin sensitive G protein. Muscarinic M_3 receptors augment phosphoinositide hydrolysis in colon (Zhang et al., 1991; Zhang and Buxton, 1991; Zhang, 1996) via coupling to a pertussis toxin insensitive G protein. In contrast, radioligand binding studies have suggested a single population of muscarinic M_2 receptors.

Muscarinic M_3 receptors mediate contraction of human colonic circular muscle (Kerr et al., 1995). Gomez et al. (1992) have identified muscarinic M_2 and M_3 receptors in both rat and human colon, although the radioligand binding data suggest that the proportions differed between the two species. Zhang (1996) has reported similar ratios in the mature rat colon tissue. The difference in these muscarinic M_2: M_3 receptor proportions between human and rat may reflect genuine species variation or post mortem changes occurring in human tissue after removal of the tissue. It is also possible that the proportions of muscarinic M_2: M_3 receptors are modulated by disease, because human HT-29 colon carcinoma cells express a homogenous population of M_3 receptors, with no evidence for M_2 receptors (Kopp et al., 1989).

In rat colon, the total number of muscarinic receptors is up-regulated after neuronal ablation by benzalkonium chloride, suggesting that these receptors are innervated (Inoue et al., 1995). Nonetheless, this ratio changes during development because, in colon of newborn rat, only muscarinic M_2 receptors, but not M_3 receptors, are detected (Zhang, 1996). These data indicate that the muscarinic M_2 receptor density is decreased and the M_3 receptor population increased as a function of age. The decrease in total muscarinic receptor population in the adult resembles that seen in guinea pig ileum (Michalek et al., 1993) and may reflect increasing parasympathetic tone on the muscle, causing progressive down-regulation (Zhang, 1996). It is unknown why the muscarinic M_2 receptor population is preferentially reduced or, indeed, what regulatory factors affect muscarinic receptor expression on smooth muscle.

Parasympathetic control of the proximal colon in the anesthetized rabbit is suggested to be mediated both by pre-junctional M_2 receptors and post-junctional M_3 receptors (Blanquet et al., 1994). By contrast, Barocelli et al. (1995) have suggested that muscarinic receptors do not, in fact, modulate contractility of isolated segments of rabbit large intestine. O'Malley et al. (1995) have shown that muscarinic M_3 receptors mediate chloride ion secretion in rat colonic epithelia. In vivo, the physiological role of post-junctional muscarinic M_2 receptors in colonic smooth muscle, or any smooth muscle from the alimentary tract, remains to be established.

3. Stomach. Post-junctional muscarinic receptors are excitatory in isolated stomach fundic strips from several species, including rabbit (Furchgott and Bursztyn, 1967; Spero, 1978), rat (Thomas and Ehlert, 1996), guinea pig (Eglen et al., 1992b) and human (Tokunaga et al., 1984). Muscarinic M_3 receptors mediate contraction of guinea pig and rat fundic muscle (Eglen et al., 1992b; Ehlert and Thomas, 1996), although most other species have not been evaluated in this respect. Isolated tissue of rat was the focus of early research on muscarinic receptor heterogeneity, because of the potential of antagonists such as pirenzepine (Hammer et al., 1980), and subsequently telenzepine (Schudt et al., 1989), to act as 'selective' gastric antisecretory agents. Hammer (1980) reported that the affinity of pirenzepine in binding studies was higher in canine fundic mucosa than that seen in fundic smooth muscle, suggesting the presence of muscarinic M_1 receptors on the gastric mucosa and M_3 receptors on the smooth muscle. However, in isolated gastric fundic cells, the binding profile of antagonists indicated the presence of a large population of muscarinic M_2 and a smaller population of M_3 receptors (Baudiere et al., 1987). Binding studies have subsequently indicated the presence of a major muscarinic M_2 receptor population and a minor population of M_3 receptors, with no evidence for the presence of M_1 receptors (Herawi et al., 1988). Similar data have been reported in human gastric smooth muscle (Bellido et al., 1995). Taken together, it is likely that muscarinic M_1 receptors, detected in the early binding studies using pirenzepine (Hammer, 1980), are probably localized to myenteric plexus ganglia. Secoverine (Zwagemakers and Claasen, 1980), in contrast to atropine, inhibits motility of mouse isolated stomach at concentrations that do not affect gastric acid secretion, suggesting antagonism of different muscarinic receptors (Davison et al., 1983). However, given the low muscarinic M_1: M_3 selectivity of secoverine (Brunner et al., 1986), this conclusion is difficult to sustain. Moreover, atropine, a non-selective muscarinic antagonist, also shows differential affinities in this preparation, because of distortion of equilibrium conditions, possibly by an uptake process (Angus and Black, 1979). This mechanism may also explain the atypical value of atropine at muscarinic receptors in guinea pig gastric fundus reported by Del Tacca et al. (1990). Studies in antral G-cells from rabbit have shown that muscarinic M_3 receptors mediate gastrin release (Weiger et al., 1994), the presence of which was corroborated by RT-PCR studies in rabbit parietal cells (Kajimura et al., 1992). In situ hybridization studies have shown the expression of muscarinic m1 mRNA in zymo-

gen cells of rat gastric corpus, with a lower level of expression in smooth muscle (Helander et al., 1996).

Relatively few biochemical studies have been undertaken in gastric smooth muscle, although Moummi et al. (1988) reported that a muscarinic receptor with low affinity for pirenzepine, (presumably muscarinic M_2 receptor), was detected in radioligand binding studies, activation of which reduced intracellular cyclic adenosine monophosphate (cAMP).

4. Gallbladder. Cholinergic innervation controls gallbladder motility, but the nature of the muscarinic receptor involved is unclear (Karahan et al., 1991). The receptor was initially thought to be dissimilar from both muscarinic M_2 and M_3 receptors (Kurtel et al., 1990), because of an atypically low affinity for 4-DAMP, atropine and pirenzepine. Ozkutlu et al. (1993), using methoctramine and *p*-F-HHSiD concluded that muscarinic M_4 receptors mediated contraction. Karahan et al. (1991) reported atypical affinity values at receptors mediating contraction of the guinea pig common bile duct, yet concluded that muscarinic M_3 receptors mediated the response. Von Schrenck et al. (1993), using these and other antagonists, showed that muscarinic M_3 receptors mediated contractions of this tissue. Barocelli et al. (1994a), in contrast, speculated that the receptor in guinea pig gall bladder differs from that in ileum, because of differential affinity of nuvenzepine.

The affinities of antagonists at muscarinic receptors mediating *both* phosphoinositide hydrolysis and inhibition of adenylyl cyclase activity in guinea pig gallbladder were consistent with activation of muscarinic M_3 receptors (von Schrenck et al., 1994; Takahashi et al., 1994b). Contractile responses of muscarinic agonists in this tissue are insensitive to pertussis toxin (von Schrenk et al., 1994). In cat gallbladder, moreover, Chen et al. (1995) have suggested that muscarinic M_2 receptors augment calcium influx via activation of phospholipase D, whereas muscarinic M_3 receptors couple to phosphoinositide hydrolysis via activation of phospholipase C (PLC). It therefore appears that the gallbladder is a smooth muscle that, although containing excitatory muscarinic receptors, remains to be fully characterized.

5. Taenia caeci. Muscarinic receptors mediate contraction of taenia caeci muscle in several species, including the guinea pig (Hobbinger et al., 1969). In this species, this occurs by activation of muscarinic M_3 receptors (Eglen et al., 1987), even though the receptor can be activated by McN-A-343. The ability of this agonist to induce contraction may reflect a high receptor reserve (Eglen et al., 1987), although a comparison of the affinity and potency for McN-A-343 does not support this suggestion (Darroch et al., 1991). It remains unclear, therefore, why McN-A-343 can act as a full (Eglen et al., 1987) or partial agonist with high intrinsic activity (0.85, Darroch et al., 1991) in this tissue.

Nonetheless, the muscarinic receptor population in this tissue is heterogeneous, because muscarinic M_3 receptors augment phosphoinositide hydrolysis while muscarinic M_2 receptors inhibit adenylyl cyclase activity augmented by isoproterenol (Elnatan and Mitchelson, 1993). Isolated smooth muscle cells from rabbit cecum also contract in response to muscarinic M_3 receptor activation, because of augmentation of phosphoinositide hydrolysis (Cuq et al., 1994). Activation of muscarinic M_2 receptors in these cells inhibit adenylyl cyclase activity (Cuq et al., 1994). Because muscarinic M_3 receptors were undetected in a radioligand binding assay, the M_2 receptor population may predominate (Cuq et al., 1994). Indeed, Elnatan and Mitchelson (1993) have shown in binding studies using guinea pig taenia that muscarinic M_2 receptors form 70% of the population.

6. Esophagus. The esophagus transports food, via peristalsis, from the pharynx to the stomach (Hendrix, 1993). A secretory function also involves the parasympathetic nervous system because, in canine epithelial mucosae, muscarinic M_3 receptors mediate increases in short circuit current, although a role for muscarinic M_1 receptors cannot be excluded (Lad et al., 1991). Muscarinic agonists elicit contraction of guinea pig (Bartlet, 1968; Kamikawa and Shimo, 1979), and rat (Bieger and Triggle, 1985) esophageal muscularis mucosae by activation of muscarinic M_3 receptors (Eglen and Whiting, 1988; Eglen et al., 1996a, b). The affinities for 4-DAMP and pirenzepine suggest that contraction of canine lower esophageal sphincter is mediated by muscarinic M_3 receptors (Goyal and Rattan, 1978; Gilbert et al., 1984). Radioligand binding, northern blot or immunoprecipitation studies in esophageal tissue from any species have not been reported, although it is unlikely that muscarinic M_3 receptors are the only subtype present. In rat isolated esophageal muscularis mucosae, a functional muscarinic M_2 population has been revealed, after depletion of muscarinic M_3 receptors (Eglen et al., 1996a). The relative proportion of M_2: M_3 receptors in this tissue is, however, unknown.

7. Anococcygeus and rectum. The rat isolated anococcygeus muscle, although lacking cholinergic innervation, responds to low concentrations of acetylcholine (Gillespie, 1972; Doggrell, 1983). Muscarinic M_3 receptors mediate contraction of this tissue, on the basis of high affinity toward 4-DAMP and a low affinity toward gallamine (Oriowo, 1983). This was supported by the high affinity of *p*-F-HHSiD at this receptor (Eglen et al., 1990b). Because the rat anococcygeus muscle lacks β-adrenoceptors (Gillespie, 1972), it is anticipated that the tissue also will lack post-junctional muscarinic M_2 receptors, although this remains to be proven. Contractions of the rectum, as with contractions in other areas of the alimentary tract, are induced by acetylcholine (Del Tacca et al., 1990). The muscarinic receptor mediating this response has not been extensively studied, but the affinities of gallamine and 4-DAMP indicate activation of post-junctional muscarinic M_3 receptors (Akah and Oriowo, 1985).

B. Respiratory Smooth Muscle

The respiratory tract receives efferent cholinergic parasympathetic innervation via the vagus nerve, stimulation of which produces rapid bronchoconstriction that is blocked by atropine (see Richardson, 1979 for review). Muscarinic receptors are widely distributed in the respiratory tract, being identified on smooth muscle, submucosal glands, epithelium, blood vessels and parasympathetic nerves (see Barnes, 1993; Pendry, 1993; White, 1995 for reviews)

Early in vitro studies in guinea pig trachea suggested that the receptors mediating contraction of tracheal smooth muscle differed from those in the atria (Barlow et al., 1972). These data, together with binding studies in swine trachea that showed low affinity for pirenzepine (Yang et al., 1986), suggested a lack of involvement of muscarinic M_1 or M_2 receptors and implicated a role for muscarinic M_3 receptors. However, the combination of binding and functional studies in bovine trachea provided conflicting results (Roffel et al., 1988) and was reminiscent of reports in guinea pig ileum. The situation was resolved in binding and northern blot studies, demonstrating that both muscarinic M_2 and M_3 receptors are expressed. Much of the early work was performed in bovine tracheal smooth muscle (Roffel et al., 1988; 1990); however, muscarinic receptor heterogeneity has been shown to be true of all species studied to date.

In those species in which the muscarinic M_2: M_3 receptor proportions of airway smooth muscle have been determined, the pharmacological profile suggests that the muscarinic M_2 receptor predominates. Radioligand binding data indicate that M_2 receptors account for approximately 70% of the muscarinic receptor population (bovine trachea, 70-80%: Roffel et al., 1988; Schaefer et al., 1995; swine trachea, 70%: Haddad et al., 1994; rabbit trachea, 80%: Mahesh et al., 1992; canine trachea, 89%: Fernandes et al., 1992; rat trachea, 70-75%: Fryer and El-Fakahany, 1990). In bovine trachea, Misle et al. (1994) have shown that muscarinic M_2 receptors are localized to the membrane fraction and are sensitive to alkylation by N-ethylmaleimide. Binding studies using isolated smooth muscle cells from canine and guinea pig trachea suggest that the proportion of M_2 and M_3 receptors may be approximately equal (guinea pig trachea 50-60% M_2, Haddad et al., 1991; canine trachea 55% M_2, Yang, 1991), whereas muscarinic M_2 receptors form 60% of the population in double-muscled calf trachea (Roets et al., 1992). Surprisingly, no data are currently available on the relative proportions of M_2 and M_3 receptors in human airway smooth muscle. However, heterogeneity is anticipated, because m2 and m3 mRNAs have been detected in human airway smooth muscle (Mak et al., 1992), and binding studies in human trachea have shown the presence of high and low affinity binding sites (Van Koppen et al., 1985). Moreover, cultured human smooth muscle cells from trachea also express functional muscarinic M_2 and M_3 receptors (Widdop et al., 1993). The relative proportions of M_2 and M_3 receptors in airway tissue, in general, may vary depending upon whether central, peripheral or whole lung tissue is used as well as the age of the animal (see Wills-Karp, 1994 for review). In lung tissue of young guinea pigs, for example, 73% of muscarinic receptors are of the M_2 subtype and 27% M_3 subtypes, whereas in older tissue, 37% were M_2 receptors, 30% were M_3 and 33% were M_1 receptors (Wills-Karp, 1993).

Despite the mixed muscarinic receptor populations in airway smooth muscle, in vitro functional studies clearly demonstrate that muscarinic M_3 receptors mediate the contractile response in this tissue (Barlow et al., 1972; Roffel et al., 1988, 1989, 1990; Eglen et al., 1990a; Brichant et al., 1990; Haddad et al., 1991; Mahesh et al., 1992; Janssen and Daniel 1990; Yu et al., 1992; Garssen et al., 1993; Loenders et al., 1992, 1994; Eltze and Galvan, 1994; Watson et al., 1995a). Biochemical studies have shown that in canine (Baron et al., 1984) and bovine (Grandordy et al., 1986; Roffel et al., 1990) trachea, or human bronchial smooth muscle (Meurs et al., 1989) or primary cultures (Widdop et al., 1993), muscarinic receptor-mediated contraction is brought about by the hydrolysis of inositol phospholipids and the generation of inositol (1,4,5)-trisphosphate ($InsP_3$) and diacylglycerol (DG). Combining information from binding, functional and biochemical studies, it is evident that, in airway smooth muscle, including human trachea, there is a large receptor reserve for contraction (Van Koppen et al., 1985; Gunst et al., 1989). Binding and functional studies in human trachea (Van Koppen et al., 1985) suggested that the affinity of methacholine is lower than the potency. Biochemical studies in human trachea (Meurs et al., 1989) have demonstrated that inositol phospholipid hydrolysis induced by methacholine has a potency that agrees with the affinity of this agonist, but is once again lower than the potency of methacholine at receptors mediating contraction (Roffel et al., 1989). These data indicate that a large receptor reserve exists for agonists mediating contraction and may serve to buffer the inhibitory effects of β-adrenergic stimulation (Gunst et al., 1989). Conversely, Ethier et al. (1996) have demonstrated a large receptor reserve associated with the muscarinic M_2 receptor that inhibits cAMP accumulation. These differences, collectively, may provide an explanation for the observation that β-adrenergic relaxations are more effective against contractions induced by leukotriene D_4, by 5-hydroxytryptamine (5-HT) or by histamine than against those induced by a muscarinic receptor agonist (Torphy, 1984; Gunst et al., 1989).

An alternative, and perhaps complementary, explanation for this observation relates to the large muscarinic M_2 receptor population, present in airway smooth muscle. Muscarinic M_2 receptors do not play a direct role in smooth muscle contraction, under normal conditions,

yet biochemical studies have consistently demonstrated muscarinic M_2 receptor-mediated inhibition of adenylyl cyclase (Sankary et al., 1988; Pyne et al., 1992; Widdop et al., 1993). Because β-adrenoceptor activation results in the stimulation of this enzyme in smooth muscle and leads to relaxation, muscarinic M_2 receptor activation may inhibit smooth muscle relaxation (Eglen et al., 1994b). This model is directly analogous to that proposed for alimentary tract smooth muscle (see above), although it remains unclear whether this is, in fact, the role of this majority muscarinic M_2 receptor population.

Muscarinic receptors are present in peripheral lung tissue (Gies et al., 1989), but it is unclear whether these are associated with airway or vascular smooth muscle because of the anatomical complexities of this tissue (Bertram et al., 1983). In rat peripheral lung, small amounts of m3 but large amounts of m2 receptor protein have been detected using immunoprecipitation techniques (Wall et al., 1991; Yasuda et al., 1993). In isolated rat lung, Post et al. (1991) have shown that muscarinic M_3 receptors mediate contraction, whereas Esqueda et al. (1996) have shown that M_3 receptors stimulate cAMP accumulation and inositol phospholipid hydrolysis. In human and guinea pig peripheral lung, a mixture of muscarinic M_1 and M_3 receptors and possibly M_2 receptors has been identified autoradiographically (Mak and Barnes 1990). mRNA for these three receptor subtypes has also been detected (Mak et al., 1992), with no evidence for muscarinic M_4 receptors in human lung. In contrast, radioligand binding (Lazareno et al., 1990), northern blot (Dorje et al., 1991a), and immunoprecipitation (Levey, 1993) studies in rabbit peripheral lung tissue suggest a high proportion of muscarinic M_4 receptors. However, contractions of rabbit lung strips are mediated by the minor population of muscarinic M_3 receptors (Vockert et al., 1993). Studies using guinea pig lung strips suggest that an M_2-like muscarinic receptor mediates contraction (Roffel et al., 1993a), although studies with additional antagonists are required to confirm this suggestion and distinguish it from the muscarinic M_4 receptor.

Muscarinic receptors are present on parasympathetic nerve terminals in the airways and on epithelium or secretory glands. Pre-junctional muscarinic auto-inhibitory receptors, originally described in guinea pig airways (Fryer and Maclagan, 1984) have been identified in the airways of several species, including humans (see Watson, 1994 for review; ten Berge et al., 1996). These receptors were originally designated as muscarinic M_2 receptors in functional studies using methoctramine and AF-DX 116. Subsequent studies have shown that the profile might not be entirely consistent with activation of this subtype and indicate the presence of muscarinic M_4 rather than M_2 receptors (Kilbinger et al., 1991, 1995). Primary cultures of parasympathetic nerves from guinea pig trachea have recently been shown (Fryer and Jacoby, 1996) to express functional muscarinic M_2 but not M_4 receptors, despite the atypical pharmacology reported previously (Kilbinger et al., 1995). Additionally, auto-inhibitory receptors also have been demonstrated in parasympathetic nerves supplying secretory submucosal glands in ferret trachea (Ramnarine et al., 1996), which are muscarinic M_2 in nature. Because muscarinic M_3 receptors mediate secretion, it is unclear what role is played by the muscarinic M_1 receptor localized to the submucosal glands (Mak and Barnes, 1990).

Contractile responses to muscarinic agonists in most airway tissue are influenced by secretory activity of the epithelium (Spina, 1994). The epithelium in guinea pig trachea may release a relaxant factor (epithelium derived relaxant factor (EpDRF)) of unknown chemical structure. The release of EpDRF has been shown to be mediated by activation of muscarinic M_3 receptors (Eglen et al., 1991), at least in guinea pig tissue. In other species, such as human trachea or bronchus, however, the release of EpDRF is equivocal, because removal of epithelium has no effect on contractile responses to carbachol, although there is a significant increase in the response to methacholine (Rabe et al., 1995; Raeburn et al., 1986). In rat trachea, Hua et al. (1994) have suggested that muscarinic M_3 receptors mediate the release of calcitonin gene-related peptide, whereas in hen trachea, muscarinic M_4 receptors may control chloride ion secretion (Winding and Bindsley, 1990).

C. Genitourinary Smooth Muscle

Most tissues of the genitourinary tract are innervated by the parasympathetic nervous system and, in most of these, post-junctional muscarinic receptors are excitatory. An overview of autonomic innervation of genitourinary tissue can be found in Maggi (1993). Muscarinic receptors in the urinary bladder have been extensively studied, because of the clinical importance of antagonizing this receptor in the treatment of urge incontinence. Moreover, the guinea pig uterus appears unique, because it is one of the few smooth muscles in which activation of muscarinic M_2 receptors mediates contraction. By contrast, the muscarinic receptor in other genitourinary smooth muscles, including the ureter, urethra, prostate and corpus cavernosum, have been poorly characterized.

1. Uterus. The uterine body from several species receives an extensive cholinergic innervation (Traurig and Papka, 1993; Tetsuro et al., 1994). Nerve stimulation-evoked contractions of the human isolated uterus are completely abolished by atropine, providing evidence for cholinergic (muscarinic) neurotransmission in nerve-evoked myometrial contractions (Morizaki et al., 1989).

In contrast to those muscles discussed above, muscarinic M_2 receptors may mediate contraction of guinea pig myometrial tissue, a conclusion based upon affinity values for hexamethonium, pirenzepine and methoctramine (Eglen et al., 1989). This proposal is supported in subsequent functional studies in which a broader range

of antagonists were studied (Bognar et al., 1992; Doods et al., 1993). Alternatively, Leiber et al. (1990) have suggested the involvement of muscarinic M_2 and M_3 receptors, based, primarily, on the finding that the AF-DX 116 concentration-effect curve for inhibition of carbachol-induced contraction of guinea pig myometrium is biphasic, an observation that awaits confirmation. Dorje et al. (1990) have suggested that muscarinic M_4, rather than M_2, receptors mediate the response, given the high affinity of sila-hexocyclium. Subsequent studies using himbacine (Doods et al., 1993) or imperialine (Eglen et al., 1992b), antagonists that discriminate muscarinic M_2 from M_4 receptors, support the notion that muscarinic M_2 receptors mediate contraction. Competition radioligand binding, (both equilibrium inhibition experiments and measurement of dissociation rates), together with northern blot studies in myometrial tissue, are consistent with a preponderance of muscarinic M_2 receptors (Eglen et al., 1989, 1992a).

In rat isolated myometrial membranes, muscarinic M_2 receptors are the only muscarinic receptor detected in radioligand binding studies (Pennefather et al., 1994), although the receptor mediating contraction in this tissue has yet to be functionally characterized. Immunoprecipitation studies in rabbit uterus have shown that muscarinic M_2 receptors form the majority, although M_3 and M_4 receptors can also be detected (Dorje et al., 1991a). The muscarinic receptor subtype mediating contraction of human myometrium has not been operationally defined. Interestingly, the affinity of secoverine at muscarinic receptors in human myometrium is lower than that at receptors in human gut muscle (Sanger and Bennett, 1981). The low selectivity of secoverine between muscarinic receptor subtypes, however, means that no firm conclusions can be made from these data.

Biochemical studies in guinea pig myometrium show that both inhibition of adenylyl cyclase and stimulation of phosphoinositide hydrolysis occurs in response to muscarinic receptor activation, the former being attributed to muscarinic M_2, and the latter to M_3 receptor activation (Marc et al., 1986; Leiber et al., 1990). Studies in the rat myometrium have also implicated a role of M_3 receptors in stimulation of phosphoinositide hydrolysis (Varol et al., 1989a, b). Contraction of guinea pig myometrium is insensitive to pertussis toxin, as is the augmentation of phosphoinositide hydrolysis (Marc et al., 1988). Taken together, the biochemical basis for muscarinic M_2 receptor-mediated contraction of guinea pig uterus remains unclear, and a role for muscarinic M_3 receptors cannot be definitively excluded.

Muscarinic receptor density and functional responsiveness appears to be influenced by the hormonal milieu and state of pregnancy. In rabbits, pregnancy induces a 61% decrease in muscarinic receptor density compared with age-matched virgin controls (Brandes and Ruggieri, 1995). However, estrogen pretreatment of rabbits does not influence muscarinic receptor-mediated phosphoinositide hydrolysis, even though the sensitivity to α_1-adrenoceptor agonists is increased (Riemer et al., 1988). In rat myometrium, as gestation progresses to term, there is a decline in muscarinic receptor-mediated phosphoinositide hydrolysis, possibly because of decreases in muscarinic receptor number (Varol et al., 1989b). Cholinergic nerves disappear from the uterine body with advancing pregnancy, consistent with the suggestion that down-regulation occurs to accommodate a reduction in the release of acetylcholine (Traurig and Papka, 1993). Again, little information is available in terms of changes in the muscarinic receptor population in animal or human myometrium during pregnancy or during the menstrual cycle: a surprising deficit, given the changing portfolio of G proteins expressed in pregnancy and parturition (see Lopez-Bernal et al., 1995 for review).

2. Urinary bladder and urethra. Activation of the cholinergic system is the major pathway by which bladder contraction, and thus voiding, is achieved in humans and primates (Taira, 1972; Hoyle and Burnstock, 1993). This may be less evident in species such as the cat or rat, where excitatory innervation is largely or partly noncholinergic (Hoyle and Burnstock, 1993).

Radioligand binding studies using [^{3}H]quinuclidinyl benzylate ([^{3}H]QNB), [^{3}H]N-methylscopolamine ([^{3}H]NMS) or [^{3}H]4-DAMP have identified a high density of muscarinic receptors in rat (Monferini et al., 1988), rabbit (Lepor and Kuhar, 1984; Batra, 1987; Levin et al., 1988; Ruggieri and Luthin, 1990), guinea pig (Nilvebrant and Sparf, 1983) and human (Nilvebrant et al., 1985; Batra et al., 1987; Levin et al., 1988; Lepor et al., 1989; Ruggieri and Luthin, 1990; Kondo et al., 1993, 1995) bladder. The lack of high affinity [^{3}H]pirenzepine binding in these tissues excludes the presence of a large population of M_1 receptors. Northern blot hybridization analysis in the rat and pig (Maeda et al., 1988) and human (Yamaguchi et al., 1994; 1996) bladder have shown the presence of mRNA encoding the M_2 and M_3 subtypes but not the M_1 or M_4 subtypes. This finding was recently corroborated by a study that showed that only the M_2 and M_3 subtypes could be immunoprecipitated from human, rat, rabbit and guinea pig bladder membranes (Wang et al., 1995). Furthermore, it was shown that the M_2: M_3 ratio was 9:1 in the rat bladder and 3:1 in the other species examined, indicating the predominance of M_2 receptors.

Pharmacological antagonist characterization of muscarinic receptors mediating contraction of detrusor muscle in rat (Wang et al., 1995; Longhurst et al., 1995; Hegde et al., 1996), rabbit (Tobin and Sjogren, 1995), mouse (Durant et al., 1991), guinea pig (Noronha-Blob et al., 1989) and human (Poli et al., 1992; Newgreen and Naylor, 1996b) bladder suggest the involvement of M_3 receptors. However, the role of the dominant M_2 receptor population is becoming clearer. Methoctramine, a selective muscarinic M_2 receptor antagonist, potently

inhibits reflex volume-induced bladder contractions in the anesthetized rat (Hegde et al., 1996). Furthermore, pretreatment with propranolol decreased the inhibitory potency of methoctramine in this model. Collectively, these findings suggest that the role of M_2 receptors in the bladder is to oppose β-adrenoceptors, activation of which facilitates bladder relaxation during urine storage. Indeed, a recent in vitro study has shown that a functional role of muscarinic M_2 receptors to reverse β-adrenoceptor-mediated relaxation of rat isolated urinary bladder can be demonstrated under certain experimental conditions (Choppin et al., in press). It can, therefore, be postulated that during bladder voiding, muscarinic M_3 receptors cause direct smooth muscle contraction, whereas M_2 receptors oppose sympathetically mediated smooth muscle relaxation. These two actions may synergize to cause more efficient discharge of urine. It remains to be seen whether this mechanism is operative in other species, including humans.

Muscarinic receptor stimulation induces phosphoinositide hydrolysis in guinea pig (Noronha-Blob et al., 1989) and human (Andersson et al., 1991) urinary bladder. The muscarinic receptor mediating this response has been pharmacologically characterized in cultured human detrusor smooth muscle cells and shown to be muscarinic M_3 receptors (Harriss et al., 1995). It is probable that the direct muscarinic M_3 receptor mediated contraction of the detrusor is a sequelae of phosphoinositide hydrolysis. Muscarinic agonists also inhibit adenylyl cyclase activity in the rabbit (Ruggieri et al., 1987) and guinea pig (Noronha-Blob et al., 1989) bladder and is the most probable mechanism by which muscarinic M_2 receptors functionally oppose β-adrenoceptor-mediated relaxation. Indeed, co-immunoprecipitation studies suggest that muscarinic M_2 and M_3 receptors couple to members of the G_i and $G_{q/11}$ families, respectively (Wang et al., 1995), resulting in inhibition of adenylyl cyclase activity and augmentation of phosphoinositide hydrolysis, respectively.

Parasympathetic nerves innervating the urinary bladder are endowed with pre-junctional inhibitory and facilitatory muscarinic receptors that are differentially activated, depending upon the frequency of nerve stimulation (D'Agostino et al., 1986, 1993; Somogyi and De Groat, 1992). The inhibitory pre-junctional muscarinic receptor has been classified as muscarinic M_2 in the rabbit urinary bladder (Tobin and Sjogren, 1995), muscarinic M_4 in the guinea pig urinary bladder (Alberts, 1995) and M_2 in the rat urinary bladder (Somogyi and De Groat, 1992). The pre-junctional facilitatory muscarinic receptor appears to be M_1 in the rat and rabbit urinary bladder (Tobin and Sjogren, 1995, Somogyi et al., 1995).

An increased incidence of urge incontinence occurs as a function of age (Rosenthal and McMurtry, 1995). Aging has been shown to either decrease (Ordway et al., 1986; Johnson et al., 1988) or increase (Latifpour et al., 1990) muscarinic receptor density in the bladder base of rats and rabbits, respectively. In humans, tissue muscarinic receptor density decreases in neurogenic (Lepor et al., 1989) and hyper-reflexic (Restorick and Mundy, 1989) bladders in comparison to tissue from normal controls. Muscarinic receptor density in rabbit bladder is decreased following short-term partial obstruction of the urethra (Levin et al., 1984). This model may have some clinical relevance because the detrusors from patients with infravesical obstruction and benign prostatic hyperplasia exhibit reduced functional responsiveness to exogenous acetylcholine (Yokoyama et al., 1991). The urinary bladder of diabetic patients show an increased density of muscarinic receptors that is accompanied by augmentation of muscarinic receptor-mediated phosphoinositide hydrolysis and contraction (Latifpour et al., 1989, 1991; Mimata et al., 1995). The relevance of this finding to the pathophysiology of diabetic cystopathy is unknown. Few data are available concerning the changes in relative proportions of muscarinic M_2 and M_3 receptors in bladder pathology or aging. The urethra has been much less well studied in comparison with the urinary bladder, although activation of muscarinic receptors causes a contraction, the magnitude of which is dependent on the species.

3. Ureter. The ureteral smooth muscle functions to transport urine from the kidneys to the urinary bladder, by induction of peristalsis. Histochemical studies have demonstrated a rich cholinergic innervation of the intravesical ureter but not the proximal ureter (Prieto et al., 1990). Cholinergic nerves are present in all three layers of the ureter, including the outer adventitia, middle smooth muscle layer and the inner mucosal layer, although the function of acetylcholine release in these layers is unclear (Amann, 1993). Little is known concerning the role of the cholinergic system and muscarinic receptors in ureteral peristalsis. In the pig isolated intravesical ureter, carbachol increases the frequency of phasic contractile activity via activation of multiple muscarinic receptors and enhances the basal tone via stimulation of muscarinic M_1 receptors (Hernandez et al., 1993). Radioligand binding in the pig intravesical ureter have shown a predominance of M_2 receptors (Hernandez et al., 1995). Morita et al. (1994) have reported that muscarinic agonists, such as carbachol, augment the occurrence of rhythmic contractions in canine ureter. The nature of the muscarinic receptor subtype mediating this response has not been investigated. Interestingly, an atypical muscarinic receptor has been identified in sheep ureterovesical junction, although it is unknown whether more than one subtype in involved in contraction (Rivera et al., 1992)

4. Prostate. The human prostate is sparsely innervated by cholinergic nerves (Dail, 1993). Acetylcholinesterase-positive fibers can be found in the fibromuscular stroma, around the acini and ducts of prostatic glands, and along blood vessels. Radioligand binding studies

have shown that the majority of muscarinic receptors in the human prostate are of the muscarinic M_1 subtype, but these are localized by immunocytochemistry to the glandular epithelium (Ruggieri et al., 1995). However, muscarinic receptor agonists stimulate contraction of isolated smooth muscle strips from human prostate capsule but not from prostatic stroma (Caine et al., 1975). Furthermore, muscarinic M_2 receptors can be detected using radioligand binding studies in primary cultures from human prostatic smooth muscle (Yazawa et al., 1994). In these cells, activation of muscarinic receptors inhibits adenylyl cyclase activity elevated by both forskolin and β-adrenoceptor agonists, in a similar manner to that found in intact prostate tissue (Shima et al., 1983). The effect, if any, of muscarinic agonists on phosphoinositide hydrolysis in these cells has not been investigated. Muscarinic M_3 receptors predominate in rat prostate, and these are down-regulated during aging (Yazawa and Honda, 1993). However, it is unclear whether these receptors mediate effects on smooth muscle tone.

5. Vas deferens, seminal vesicle, testis, and epididymis. Muscarinic agonists cause contraction of vas deferens from several species, although the muscarinic subtype involved varies among the species. In the dog, the density of muscarinic receptors is highest in the prostatic portion and lowest in the epididymal portion, possibly reflecting different levels of parasympathetic innervation (Konda et al., 1994). In the rat, Doggrell (1986) has suggested that contractions of the epididymal portion are mediated by muscarinic M_1 and M_2 receptors, whereas a recent study has implicated the involvement of muscarinic M_3 receptors in contraction of the whole vas deferens (Miranda et al., 1994). Radioligand binding studies in rat vas deferens suggest a predominance of muscarinic M_2 receptors (Kamai et al., 1994). In the human vas deferens, muscarinic M_1 receptors mediate contractile responses to exogenous acetylcholine (Miranda et al., 1992). In the rabbit vas deferens, muscarinic M_2 receptors mediate potentiation of neurogenic contractions, whereas in the mouse vas deferens, muscarinic M_3 or M_4 receptors mediate a similar effect (Matsuno and Mita, 1992).

Few studies have characterized the cholinergic innervation of the seminal vesicle, although most studies locate these neurons to the epithelium (Gonzales, 1989), with the smooth muscle receiving little or no innervation (Dail, 1993). However, Al-Zuhair et al. (1975) have reported that a rich cholinergic plexus is present in the inner circular muscle layer in guinea pig seminal vesicle. Contractions of this tissue are mediated by activation of muscarinic M_3 receptors, and northern blot studies have failed to find evidence for expression of muscarinic m2 mRNA (Eglen and Harris, 1993b).

Autonomic innervation plays only a minor role in the control of the testis (Hodson, 1970), and cholinergic innervation of this tissue is sparse or absent. Muscarinic receptors may mediate contraction of the smooth muscle capsule, although the subtype is undefined. In terms of the epididymis, the extent of cholinergic innervation varies according to species and location.

6. Corpus cavernosum. The parasympathetic nervous system has been proposed to play an important role in tumescence and penile erection by contributing to relaxation of corpus cavernosum smooth muscle (Anderson, 1993). Intracavernous injection of acetylcholine in adult male dogs produces increases in intracavernous pressure accompanied by sustained erection (Takahashi et al., 1992). Exogenous acetylcholine causes relaxation of pre-contracted human and rabbit corpus cavernosum via muscarinic receptor-mediated release of nitric oxide from endothelial cells (Saenz de Tejada et al., 1988; Knispel et al., 1992). Radioligand binding studies have shown the presence of muscarinic M_3 receptors on the human corpus cavernosum and endothelial cells derived from this tissue, suggesting the involvement of this receptor in relaxation of smooth muscle (Traish et al., 1990). In addition, in situ hybridization studies have shown the presence of muscarinic M_2 and M_4 receptor mRNA in smooth muscle cells of the human corpus cavernosum (Toselli et al., 1994; Traish et al., 1996), although the precise role of these receptors in direct modulation of smooth muscle tone is unclear.

D. Ocular Smooth Muscle

Cholinergically innervated smooth muscles play an important role in regulating both the amount of light entering the eye (iris) and the point on the retina at which it is focused (ciliary body). The iris regulates the amount of light entering the eye and comprises radially arranged smooth muscle (dilator pupillae), which is sympathetically innervated, and concentrically arranged smooth muscle (constrictor pupillae or iris sphincter), which is parasympathetically innervated. It is well established that acetylcholine, via activation of muscarinic receptors, regulates pupillary diameter via activation of pre- and post-junctional receptors (see Fuder, 1994 for review). Indeed, a significant side effect of non-selective muscarinic receptor antagonists is the occurrence of mydriasis, testimony to the strong parasympathetic tone.

In cultures of human iris sphincter, muscarinic M_3 receptors predominate, as judged by the high affinity binding of 4-DAMP to a single population of sites (Woldemussie et al., 1993). At these sites, the affinity of pirenzepine and AF-DX 116 is indicative of a muscarinic M_3 receptor (Woldemussie et al., 1993). Erikson-Lamy et al. (1991), however, have shown that mRNAs encoding both m2 and m3 receptors were present, suggesting that muscarinic M_2 receptors, if present, are below the detection limit of binding assays. Northern blot analysis in bovine iris sphincter muscle has shown m3 mRNA is predominantly expressed, with only minor amounts of m2 mRNA (Honkanen et al., 1990). In human ciliary

muscle cell culture, muscarinic M_3 receptors mediate contraction and augmentation of PLC activity (Pang et al., 1994). Muscarinic M_3 receptors also appear to mediate contraction of ciliary muscle strips from rhesus monkeys (Poyer et al., 1994). In anesthetized cats, McN-A-343 induces pupillary contraction by activation of post-junctional muscarinic M_3 receptors in the iris sphincter (Koss and Wally, 1995). The role of other muscarinic receptor subtypes in this tissue has not been extensively studied.

At muscarinic receptors mediating contraction, the affinities for pirenzepine, 4-DAMP and *p*-F-HHSiD are consistent with M_3 receptor activation (Matsumoto et al., 1994). These findings support earlier data by Barlow et al. (1972) showing that the muscarinic receptor subtype mediating contraction of guinea pig iris sphincter resembled that in ileum and trachea. Shiraishi and Takayanagi (1993) reported that muscarinic M_3 receptors mediate both contraction *and* relaxation of rat iris dilator smooth muscle. In rat iris dilator muscle, muscarinic M_3 receptors mediate relaxation at low agonist concentrations but contraction at higher concentrations (Shiraishi and Takayanagi, 1993; Masuda et al., 1995). These responses are mediated by two distinct G proteins, one of which (relaxation) is sensitive to pertussis toxin (Yamahara et al., 1995). Several studies suggest that activation of muscarinic receptors in bovine, cat and rabbit iris muscle inhibit adenylyl cyclase activity (Abdel-Latif et al., 1992; Tachado et al., 1994), although the nature of this subtype has not been defined.

Taken together, the muscarinic M_3 receptor in iris smooth muscle exhibits pleiotropic coupling to G proteins and is the predominant receptor subtype present. In this respect, the tissue is one of the few exceptions in tissues studied to date that lack muscarinic M_2 or M_4 receptors, through which adenylyl cyclase activity is inhibited. Pharmacologically, canine iris muscle may also possess an atypical muscarinic M_3 receptor. Although unconfirmed, preliminary data by Wallis et al. (1995), show that zamifenacin exhibits an affinity (pK_B) of less than 6.0 at receptors mediating contraction of isolated iris muscle and an affinity (pK_B) of 8.6 at muscarinic M_3 receptors mediating contractions of canine isolated ileum. Additional contractile and biochemical studies are clearly required to define the nature of the muscarinic receptor population in canine iris tissue.

The muscarinic receptor mediating contraction of rabbit isolated iris muscle is also ill-defined (Honkanen and Abdel-Latif, 1988). It is unlikely that muscarinic M_1 receptors mediate contraction, because pirenzepine exhibits an intermediate affinity and AF-DX 116 a low affinity at receptors mediating phosphoinositide hydrolysis, myosin light chain phosphorylation and contraction (Akhtar et al., 1987). The affinity values for *p*-F-HHSiD, AQ-RA 741 and UH-AH 37, however, were inconsistent with activation of muscarinic M_1, M_2, M_3 and M_4 receptors (Bognar et al., 1992). Further studies clearly are required to define the nature and, indeed, the number of muscarinic receptor subtypes involved in contraction, because changes in rabbit intraocular pressure are extensively used as a screen to identify novel muscarinic agonists for the treatment of glaucoma.

E. Vascular Smooth Muscle

Acetylcholine can contract and relax vascular tissue, with the latter effect being principally mediated by the release of relaxant factors such as nitric oxide from the endothelium. The literature in this field was reviewed previously by our group (Eglen and Whiting, 1990). In general, muscarinic M_3 receptors mediate endothelium-dependent relaxation, whereas contraction can be mediated via several subtypes. Vascular relaxation may also be mediated by endothelial-independent mechanisms, depending upon the anatomical location of the vessel. Recent reports have shown that muscarinic M_3 receptors mediate relaxation of equine coronary artery (Obi et al., 1995), rat renal vasculature (Eltze et al., 1993), spontaneously hypertensive rat aorta (Boulanger et al., 1994), rabbit aorta (Jaiswal et al., 1991; Watson and Eglen, 1994b), rat mesenteric bed (Hendricks et al., 1993), guinea pig uterine artery (Jovanovic et al., 1994), cat femoral artery (Fernandes et al., 1991), cat cerebral arteries (Dauphin et al., 1994), simian coronary arteries (Ren et al., 1993) and several isolated bronchial arteries (see O'Rourke and Vanhoutte, 1992 for review). Muscarinic M_3 receptors also mediate relaxation of human isolated pulmonary arteries (Norel et al., 1996) and vasodilation in the forearm of healthy volunteers or patients with essential hypertension (Bruning et al., 1994; 1995). In vessels of rat isolated lung, muscarinic M_1 and M_2 receptors mediate indirect and direct vasodilation, respectively (Wilson et al., 1995).

The muscarinic receptors mediating contraction of vascular tissue differs according to species and anatomical location. Muscarinic M_3 receptors mediate contraction of the rat coronary vascular bed (Su and Narayanan, 1993), the spontaneously hypertensive rat aorta (Boulanger et al., 1994) or rabbit aorta (Watson and Eglen, 1994b), and the simian coronary artery (Ren et al., 1993), equine coronary artery (Obi et al., 1995) and human isolated pulmonary arteries (Norel et al., 1996). This subtype also mediates contraction of guinea pig isolated portal vein (Pfaffendorf and van Zwieten, 1993). Pharmacological data suggest that contraction of canine isolated femoral and saphenous veins and of cat cerebral arteries are mediated by muscarinic M_1 receptors (Eglen et al., 1990b; O'Rourke and Vanhoutte, 1987; Dauphin and Hamil, 1992). Muscarinic M_1 receptors may also mediate contraction of rabbit pulmonary circulation (El-Kashef and Catravas, 1991), although this remains to be substantiated with a range of antagonists.

One confusing aspect concerning muscarinic receptors in vascular endothelial cells was the lack of specific binding sites for [^{3}H]QNB or [^{125}I]-QNB (Stephenson et

al., 1988), even though functional responses can be clearly demonstrated (see Eglen and Whiting, 1990, for review). Although culturing conditions can suppress the expression of muscarinic receptors (Tracey and Peach, 1992), freshly cultured corporal endothelial cells express either muscarinic M_1 or M_3 receptor mRNA (Traish et al., 1994). Binding studies (Dauphin and Hamel, 1992; Dauphin et al., 1994) in human and cat pia-arachnoid vessels reveal the presence of muscarinic receptors in smooth muscle, with muscarinic M_1 receptors forming 40% (human) and 20% (cat) of the total number of sites. In the cat, muscarinic M_2 receptors formed 35% of the total, although this subtype was undetected by binding studies in human vessels. Muscarinic M_3 receptors formed 35% of the total binding in both species (Dauphin and Hamel, 1992; Dauphin et al., 1994). In human and bovine cerebral microvessels, muscarinic M_1 receptors have been detected (Garcia-Villalon et al., 1991), and in cerebral capillaries, both muscarinic M_1 and M_3 receptors have been detected (Linville and Hamel, 1995). It should be noted that the location of these binding sites is unclear, because [^{3}H]QNB may also bind to astrocytes present in preparations of isolated microvessel membranes (Moro et al., 1995).

The identification of muscarinic receptors in cat (Dauphin et al., 1994) microcirculatory vessels is consistent with the ability of these receptors to mediate vasoconstriction. Linville and Hamel (1995) have also identified a muscarinic receptor in human and bovine brain capillaries that regulates blood flow, via release of nitric oxide (Wang et al., 1994). It is probable that several muscarinic receptor subtypes regulate both local cerebral blood flow in concert and blood brain barrier permeability (see Dauphin and MacKenzie, 1995 for review), although the precise role of each subtype remains to be established.

F. Summary

The literature thus presents a consistent picture of muscarinic receptor function in smooth muscle. The majority of these tissues contract in response to muscarinic M_3 receptor activation, yet possess a large proportion of muscarinic M_2 receptors. The majority of these studies are summarized in table 8. Moreover, the relative proportions of M_2: M_3 receptors in the ratio of 4:1 is generally consistent across species and tissues. These two muscarinic receptor subtypes clearly couple to different G proteins, based upon their differential sensitivity to pertussis toxin, with the muscarinic M_2 receptor inhibiting adenylyl cyclase activity and the M_3 receptors augmenting phosphoinositide hydrolysis. Griffin and Ehlert (1992) proposed that muscarinic M_2 receptors act to inhibit muscle relaxations induced via elevation in intracellular cAMP. This hypothesis, subsequently developed by this group, and the growing body of supporting experimental data, is discussed below.

The finding that these two signaling systems are regulated in smooth muscles, even though only a single muscarinic receptor subtype is expressed, attests to their importance in controlling smooth muscle tone. Although promiscuous coupling of recombinant muscarinic receptors to different G protein-coupled receptors is a recognized phenomena (Lai et al., 1991), definitive evidence with endogenously expressed receptors is sparse. The iris muscle (Masuda et al., 1995), and perhaps the gallbladder (von Schrenck et al., 1993; 1994; Takahashi et al., 1994b), appear to be tissues that predominantly express muscarinic M_3 receptors that promiscuously couple to both a pertussis toxin-sensitive and insensitive G protein. These muscles, together with muscarinic M_2 receptors in rat isolated atria (Kenakin and Boselli, 1990) and cerebellum (Matesic et al., 1991) or M_3 receptors in rat parotid gland (Dai et al., 1991) collectively represent systems in which endogenous muscarinic receptors display pleiotropic coupling. In smooth muscle, the physiological consequences of the phenomena, however, and the regulation of 'channeling' of the response via a single signaling pathway is unexplored.

IV. Signal Transduction Systems and Muscarinic Receptors in Smooth Muscle

Activation of post-junctional muscarinic receptors in smooth muscle results in a series of signaling events, temporally related to phases of muscarinic receptor-mediated contraction and relaxation. These pathways also affect the ionic permeability of the membrane and thus regulate the prevailing membrane potential in a coordinated fashion. In general, the degree of muscle tone can be viewed as a dynamic balance between contractile and relaxant forces, as a result of both parasympathetic and sympathetic innervation, respectively. Classically, signaling pathways in smooth muscle are considered in terms of the muscarinic receptor (phosphoinositide hydrolysis) and β-adrenoceptors (via adenylyl cyclase activity). Muscarinic receptors may also induce smooth muscle relaxation directly by opening of potassium channels. A considerable literature now exists, however, that suggests that muscarinic receptors also modulate adenylyl cyclase activity and thus sympathetic relaxant tone. This section will discuss the evidence for an indirect role of muscarinic receptors in the control of muscle tone. Because the majority of studies have characterized muscarinic receptor subtypes in smooth muscle in terms of the regulation of phosphoinositide hydrolysis and inhibition of adenylyl cyclase activity, these signaling pathways will be emphasized.

A. Phosphoinositide Hydrolysis Regulation and Smooth Muscle Contraction

Contraction of most smooth muscles is thought to involve coupling of the muscarinic receptor, via a guanine nucleotide binding protein (G protein) to stimulation of phosphoinositide-specific PLC, inducing genera-

tion of $InsP_3$ and DG (see Fisher, 1995 for review). Studies have shown in several peripheral smooth muscles that this is mediated by activation of the muscarinic M_3 receptor via coupling to a pertussis toxin-insensitive G protein, potentially $G_{q/11}$. The generation of $InsP_3$ leads to the release of calcium from stores in the sarcoplasmic reticulum (Somlyo and Himpens, 1989). This process ultimately results in contraction, via stimulation of actin/myosin adenosine triphosphatase (ATPase), but also initiates several pathways, secondary to elevations in intracellular calcium, including stimulation of adenylyl cyclase and guanylyl cyclase. (Extensive reviews of muscarinic receptors and signaling in smooth muscle are published elsewhere; Schramm and Grunstein, 1992; Malarkey et al., 1996).

It is generally assumed that generation of $InsP_3$ leads to a rapid phase of muscle contraction, because the peak generation of $InsP_3$ precedes the initiation of contraction. In most smooth muscles, this response is mediated by activation of muscarinic M_3 receptors, and, in bovine trachea, occurs within 2 seconds of receptor activation. In tissues such as the ileum, the initiation of contraction of longitudinal smooth muscle may also be independent of $InsP_3$ generation, and it is possible that calcium influx is mediated by other second-messengers, including cyclic adenosine diphosphate (ADP) ribose or activation of phospholipase A_2 (Wang et al., 1993). The sustained phase of muscle contraction may be via a different signaling pathway, including activation of protein kinase C, after diacylglycerol formation (Nishizuka, 1986). This view is widely invoked for airway or vascular smooth muscle, in which a tonic contracture is physiologically relevant because, in contrast to the transient nature of $InsP_3$ generation, the formation of diacylglycerol is sustained. The main target of diacylglycerol is the activation of the protein kinase C (PKC) family, which exist in at least ten isoforms (Hug and Sarre, 1993). The phosphorylation of several contractile proteins by various isoforms of PKC in smooth muscle plays a key role in muscle contraction (see Malarkey et al., 1996 for review).

A research challenge in the area of muscarinic receptor control of smooth muscle tone is to explain how the release of $InsP_3$ or DG is temporally related to responses in tissues that do not sustain a tonic contracture, i.e., phasically active tissues, including the intestine, uterus, portal vein or vas deferens. Indeed, it is not presently clear how the intracellular calcium ion concentration is regulated during the spontaneous electrical and mechanical activity of many smooth muscles, although several models have been proposed (Petersen and Wakui, 1990; Post and Hume, 1992; see Sanders, 1992 for review). Janssen and Sims (1994) have suggested that oscillations of intracellular calcium leads to transient, sporadic and spontaneous Ca^{2+}-K^+ currents and, eventually, myogenic oscillations.

PLC activity per se is subject to feedback modulation in a positive and negative manner (see Fisher, 1995 for review). Thus, increase in intracellular calcium augments the activity of PLC, notably PLC-β in smooth muscle, although PLC-δ also may be involved. Blayney et al. (1996) have shown that in porcine vascular smooth muscle, six isoforms of PLC are expressed and that the activity of PLC-β_3 can be inhibited by liberation of $\beta\gamma$ subunits released from a pertussis toxin-sensitive G protein. Conversely, the activity of this isozyme can be enhanced by free α subunits liberated from G_q. PLC-β_2 activity is inhibited by cAMP-dependent protein kinase A, thus providing a potential means for cross-talk between signaling pathways involving cAMP generation and phosphoinositide hydrolysis (Liu and Simon, 1996). It remains to be seen whether this occurs in smooth muscle. PKC activation may also oppose stimulation of PLC (Lai and El-Fakahany, 1988). In canine proximal colon tissue, this occurs in a fashion independent of muscarinic receptor coupling to the G protein (Zhang and Buxton, 1993). Activation of PLC is also augmented by the influx of calcium as a result of membrane depolarization (Fisher, 1995).

B. Adenylyl Cyclase Regulation and Smooth Muscle Contraction

A common feature of muscarinic receptor activation in smooth muscle is the inhibition of adenylyl cyclase activity, via coupling of the receptor to a pertussis toxin-sensitive G protein. In most tissues, inhibition of adenylyl cyclase is mediated by activation of muscarinic M_2 as opposed to M_4 receptors. However, it is arguable that, given the poor discrimination of most antagonists between muscarinic M_2 and M_4 receptors (Caulfield, 1993), additional studies need to be done to confirm this characterization. There are at least eight forms of adenylyl cyclase and, while it is not well established which isoforms are expressed in smooth muscle, it is probable that these include the type II and type IV isoforms (Yoshimura and Cooper, 1993). The intracellular effects of cAMP, generated by activation of adenylyl cyclase, involve activation of the protein kinase A family, which causes relaxation by reducing intracellular Ca^{2+} concentration and may in turn inhibit phosphoinositide hydrolysis.

Candell et al. (1990) postulated that muscarinic M_2 receptors in rat ileum, by inhibiting agonist-induced elevations in intracellular cAMP, offset relaxations to agonists that induce relaxation via elevations of intracellular cAMP (Berridge, 1975). In rat or guinea pig ileum, muscarinic M_2 receptors inhibited cAMP accumulation induced by β-adrenoceptor agonists and forskolin, via a pertussis toxin-sensitive G protein (Candell et al., 1990; Griffin and Ehlert 1992; Reddy et al., 1995). A potential role of muscarinic M_2 receptors at inhibiting relaxation is consistent with functional studies in guinea pig, bovine and rabbit trachea. Thus, muscarinic

M_2 antagonists, such as AF-DX 116 (Fernandes et al., 1992) or methoctramine (Watson and Eglen, 1994a; Watson et al., 1995b, c; Schramm et al., 1995), augment the relaxant potency of isoprenaline in tissues pre-contracted with a muscarinic receptor agonist. Concordantly, relaxant responses to isoprenaline in canine trachea (Mitchell et al., 1993) or guinea pig ileum (Thomas and Ehlert, 1996) are enhanced by pretreatment with pertussis toxin. These findings suggest that activation of muscarinic M_2 receptors opposes the relaxant potency of isoprenaline. Moreover, the relaxant potency of β-adrenoceptor agonists is greater in trachea pre-contracted with leukotriene D_4 than in tissues pre-contracted with a muscarinic receptor agonist (Torphy, 1984; Van Amsterdam et al., 1989).

Direct, and more convincing evidence to support an inhibitory role of muscarinic M_2 receptors has come from functional studies in which the muscarinic M_3 receptor population is depleted by alkylation using 4-DAMP mustard under conditions of muscarinic M_2 receptor protection. Conditions to unmask a functional M_2 receptor have been reported (Thomas et al., 1993; Reddy et al., 1995) in tissues pre-contracted with histamine and relaxed by an agent that elevates cAMP, such as forskolin (Thomas et al., 1993) or isoprenaline (Reddy et al., 1995). Under these conditions, addition of a muscarinic agonist activates the residual muscarinic M_2 receptors and induces a reversal of the relaxant tone ('recontraction;' see Eglen et al., 1994a for review). An advantage of this procedure is that it allows pharmacological characterization of the muscarinic M_2 receptor, without the interference of a functional M_3 population, through which contractions would ordinarily be mediated. This approach is compromised by a residual muscarinic M_3 population, manifested as either biphasic agonist concentration-recontraction curves (Thomas and Ehlert, 1996) or antagonist affinities that reflect activation of more than one receptor (Choppin et al., in press; Thomas and Ehlert, 1996). Consequently, the approach is optimal in tissues with a low muscarinic M_3 receptor reserve, because it is easier to deplete by alkylation. In rat isolated esophagus, a tissue with a low muscarinic M_3 receptor reserve, recontractions to muscarinic M_2 stimulation are exclusively mediated by muscarinic M_2 receptors (Eglen et al., 1996a).

Muscarinic M_2 receptors have been shown to mediate contractile responses in guinea pig ileum (Thomas et al., 1993; Reddy et al., 1995), trachea (Thomas and Ehlert, 1996), rat urinary bladder (Choppin et al., in press; Hegde et al., 1996) and esophagus (Eglen et al., 1996a). In these studies, the relaxing agent has been either forskolin (adenylyl cyclase activation) or isoprenaline (via β-adrenoceptors). In rat esophagus, muscarinic M_2 receptors offset relaxations to 5-HT (via 5-HT_4 receptors). Collectively, these findings may argue for a general role for muscarinic M_2 receptors at inhibiting cAMP formation, and thus relaxation, induced by agents that augment adenylyl cyclase activity. Indeed, in guinea pig ileum, muscarinic M_2 receptors inhibit adenylyl cyclase activation induced by prostaglandin E_1 or E_2, 5-HT or vasoactive intestinal peptide (Reddy et al., 1995), although functional studies that correlate with these data have not been reported.

One emerging feature of current research is that demonstration of a functional role for muscarinic M_2 receptors depends on the experimental approach used. In some smooth muscles, even though expressing a large proportion of muscarinic M_2 receptors, disclosure of a post-junctional functional role for muscarinic M_2 receptors is difficult, even when muscarinic M_3 receptors are extensively depleted. In guinea pig trachea (Watson et al., 1995c) or esophagus (Watson et al., 1995d), under these conditions, muscarinic M_2 receptors are not revealed when tissues are relaxed with a β adrenoceptor agonist, yet are disclosed when the tissues are relaxed with forskolin (Thomas and Ehlert, 1996). These studies suggest that the level of cAMP generation may determine the magnitude of the muscarinic M_2 receptor response. In general, moreover, adenylyl cyclase activity is higher in the presence of forskolin than in the presence of G protein receptor activation. Physiologically, this implies that the inhibitory role of muscarinic M_2 receptors is greatest when the relaxant tone dominates (via adenylyl cyclase activity). Additional studies are required to substantiate this suggestion in vitro or in vivo.

In tracheal smooth muscle, the situation may be further complicated, because a role for muscarinic M_3 receptors in antagonism of relaxant responses to β-adrenoceptor agonists has been argued (van Amsterdam et al., 1989). In bovine trachea, a positive correlation exists between the ability of full and partial agonists to augment phosphoinositide hydrolysis and inhibition of β-adrenoceptor-mediated relaxation (van Amsterdam et al., 1989). It has been suggested that activation of PKC by muscarinic M_3 receptors uncouples the β-adrenoceptor from the G protein (Roffel et al., 1994a, b; 1995). This hypothesis is based on data from blood lymphocytes of asthmatic patients after allergenic provocation (Meurs et al., 1987), but has not been proven in airway tissue. By contrast, in recombinant CHO cells, cAMP accumulation via activation of β_2-adrenoceptors is enhanced when muscarinic M_3 receptors are co-expressed (Ellis et al., 1996) via a protein kinase C-independent mechanism (Stanford et al., 1996). Moreover, in bovine colon, this PKC activation opposes muscarinic M_3 receptor-mediated $InsP_3$ generation and does not influence the number or affinity of muscarinic M_2 receptors (Zhang and Buxton, 1993). Additional studies are thus required to demonstrate that the cross-talk, postulated by Roffel et al. (1994b, 1995), occurs in smooth muscle.

The inability of phorbol ester to mimic the effects of methacholine in airway smooth muscle does not support a PKC-dependent pathway (Pyne et al., 1993). Nonetheless, it may be that regulation of the function of G_s, but

not G_i, activity by phosphorylation is a potential mechanism of cross-talk in airway, and perhaps other, smooth muscles. Thus, in airway smooth muscle, evidence is available to link muscarinic receptors (M_2 and or M_3) and G_s function. In this model, the free $\beta\gamma$ subunits, released from coupling of muscarinic receptors to G_i or $G_{q/11}$ coupling, can associate with $G_{s\alpha}$ (Pyne and Pyne, 1994) (fig. 3).

In vascular tissue, the main role of muscarinic M_3 receptors is to enhance the release of nitric oxide, endothelial-derived relaxant factor (see Eglen and Whiting, 1990 for review), endothelial-derived hyperpolarizing factor (Hammarstrom et al., 1995), or other agents that relax smooth muscle (Eglen and Whiting, 1990). In the endothelium, β-adrenoceptors positively couple to adenylyl cyclase (Malarkey et al., 1996) and may thus increase nitric oxide production (Zhang, 1996). In this case, both muscarinic M_3 and β-adrenoceptors are involved in the production of nitric oxide. This may be one reason why muscarinic M_2 receptors have not been detected in vascular endothelium because, in contrast to other types of smooth muscle, activation of muscarinic M_3 and β-adrenoceptors both cause relaxation.

C. Ion channels and Smooth Muscle Contraction

A review of ion channels and smooth muscle function has recently been published (Aaronson and Smirnov, 1996). In guinea pig ileum, acetylcholine increases an inward cation current and thus induces action potential discharge (Lim and Bolton, 1988). This occurs via entry of sodium, a process accelerated by calcium ions and modulated by a pertussis toxin-sensitive G protein (see Bolger et al., 1989; Inoue and Isenberg, 1990; Bolton and Lim, 1991 for review). Zholos and Bolton (1994) suggest that, in guinea pig ileum, the opening of this putative cationic channel requires at least the binding of one G protein α subunit and enhancing receptor activation increases the probability of channel opening and thus depolarization. The ability of pertussis toxin to abolish the response (Prestwich and Bolton, 1995) may suggest a role for either the muscarinic M_2 or M_4 receptor in addition to regulation of adenylyl cyclase activity. The pharmacology of this receptor is consistent with activation of an N_2 receptor (Zholos and Bolton, in press). Once the smooth muscle membrane undergoes an initial depolarization, voltage-sensitive calcium channels open, and a large influx of calcium occurs. The restoration of the membrane potential occurs by opening of a calcium-dependent potassium conductance and consequent hyperpolarization. Several smooth muscles, including vascular and airway smooth muscle (see Janssen and Sims, 1994 for references), exhibit spontaneous and transient currents. These reflect activation of Ca^{2+}-dependent-K^+ currents arising from spontaneous and sporadic release of internally sequestered calcium. In tracheal myocytes, these currents are activated by muscarinic receptors (Janssen and Sims, 1992, 1994), although the nature of the muscarinic receptor subtype involved has not been established.

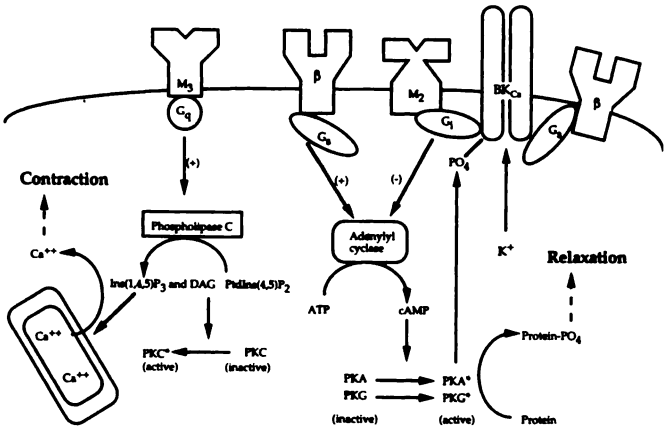

FIG. 3. A model of intracellular signaling and cross-talk of postjunctional muscarinic receptors in smooth muscle. PKC, protein kinase C; PKA, protein kinase A; DAG, diacylglycerol; BK_{Ca}, high conductance Ca^{2+}-activated K^+ channel.

Caulfield (1993) has speculated that muscarinic M_3 receptors, operating at high concentrations of agonist, evoke contractions via $InsP_3$ mobilization and calcium release. Muscarinic M_2 receptors, alternatively, evoke an inward calcium current, at lower agonist concentrations (Parekh and Brading, 1991). This hypothesis also remains to be proven using subtype-selective antagonists, but it could provide an additional role of muscarinic M_2 receptors in smooth muscle.

In some muscles, the possibility of cAMP-independent relaxant mechanism exists as a consequence of activation of β-adrenoceptors. There is increasing evidence to suggest that β-adrenoceptors may mediate relaxation via direct coupling to Ca^{2+}-dependent K^+ channels (Torphy, 1994). These channels have been identified in several smooth muscles, including respiratory (McCann and Welsh, 1986), ureter (Shuba, 1981), intestinal and arterial tissue (Benham et al., 1984; Bolton et al., 1984; Inoue et al., 1985). It has been shown in airway smooth muscle that activation of these high conductance Ca^{2+}-dependent K^+channels contributes significantly to the underlying mechanism of β-adrenoceptor activation (Jones et al., 1990; Miura et al., 1992; Huang et al., 1993). β-adrenoceptor agonists increase the activity of this channel via a G_s coupled process (Kume et al., 1992), causing membrane hyperpolarization, inhibition of Ca^{2+} influx, and thus relaxation. Importantly, muscarinic M_2 receptors inhibit this channel, via G_i, thereby inhibiting the cAMP-independent component of the β-adrenoceptor relaxation (Kume et al., 1991). Indeed, in passively sensitized rabbit airway smooth muscle, β-adrenoceptor-mediated relaxation is attenuated by an up-regulation of muscarinic M_2 receptors and G_i protein expression and coupling (Hakonarson et al., 1995). Recent data have revealed an involvement of the high conductance Ca^{2+}-activated K^+ channel in the interac-

tion between muscarinic M_2 and β-adrenergic receptors (Hakonarson et al., 1996).

V. Therapeutic Compounds in Smooth Muscle Pathology

An important therapeutic indication for muscarinic receptor antagonists is to relax smooth muscle, with the degree of relaxation produced depending upon the level of pre-existing cholinergic tone. In general, muscarinic M_3 receptors appear to mediate contraction of most types of smooth muscle studied in detail to date. Selective blockade of muscarinic M_3 receptors, therefore, should be therapeutically useful in the treatment of respiratory disorders, such as chronic obstructive pulmonary disease (COPD), gastrointestinal disorders, such as irritable bowel syndrome and urinary tract disorders, such as incontinence. The advantage of such compounds lies in the potential for reduced incidence of side effects, including blurred vision, increased heart rate, heat intolerance, sedation and mild confusion (Feinberg, 1993). These effects, uncomfortable in the young, may be serious in the elderly, because they are exacerbated with age. For example, dry mouth in the aged leads to mucosal damage, denture misfit or caries as well as upper respiratory infection caused by the loss of the antimicrobial action of saliva.

A. Gastrointestinal and Lower Urinary Tract

Selective muscarinic M_1 antagonists such as pirenzepine have therapeutic utility in the treatment of peptic and duodenal ulcers. Non-selective muscarinic antagonists, including the novel compounds, cimetropium and octylonium, have been used in the treatment of motility disorders such as irritable bowel syndrome. However, there is no convincing evidence that this class of drugs is more effective than placebo in this condition. In terms of inducing gastrointestinal smooth muscle relaxation, several relatively old compounds are available, including dicyclomine, pinaverium, fendoverine, mebeverine and milverine (see Eglen and Watson, 1996, for review). Although lacking selectivity for muscarinic M_3 receptors, they possess other properties, including calcium channel blockade, a property that will contribute to antispasmodic actions (Downie et al., 1977). Recently, selective muscarinic M_3 antagonists, such as zamifenacin (the development of which is now discontinued) or darifenacin (Phase 3 clinical trial) have been developed, that show apparent gut selectivity in animal models (Sawyer et al., 1996).

Muscarinic receptor antagonists are now recommended as a main therapy for the treatment of detrusor instability or urge incontinence (see Hieble et al., 1995 for review; Resnick, 1995). Of these, oxybutynin and propantheline are indicated for front- and second-line therapy, respectively. Tolterodine, a novel, but non-selective muscarinic receptor antagonist, is in advanced clinical trials for the treatment of urge incontinence (Nilvebrant et al., 1994). This compound may possess marginal selectivity for the urinary bladder over the salivary gland, although the underlying mechanism is unclear (Gillberg et al., 1994). Vamicamide is another compound that possesses slight (two- to four-fold) selectivity for the muscarinic M_3 receptor over M_1 and M_2 receptors (Yamamoto et al., 1995). The bladder-selective actions of this and several analogues of oxybutynin (Carter et al., 1991; Kaiser et al., 1992; 1993; Howell et al., 1994) in vivo may be caused by accumulation in the urinary bladder (Oyasu et al., 1994). A specific uptake system has been assumed in the mouse urinary bladder (Durant et al., 1991). It is unknown whether this system operates in human bladder or to what extent it affects antagonist potency in vivo. Darifenacin, the most selective muscarinic M_3 antagonist identified to date, is being clinically evaluated for the treatment of urge incontinence (Wallis et al., 1995; Swami and Abrams, 1995). This compound, because of a low affinity at muscarinic M_2 receptors, is expected to be devoid of cardiac side effects associated with other muscarinic antagonists. Like tolterodine and vamicamide, darifenacin has marginal (two- to three-fold) selectivity for the urinary bladder over the salivary gland in animal models (Newgreen et al., 1995), although it is unclear whether these findings are clinically relevant.

B. Respiratory Tract

Because cholinergic neural mechanisms may contribute to airway narrowing in asthma and COPD, muscarinic receptor antagonists are effective in treating acute bronchoconstriction, particularly in COPD (Gross and Skorodin, 1984; Doods, 1992). Antagonists currently in use for the treatment of this condition are not selective. Moreover, it has been speculated, but not proven, that a non-selective muscarinic receptor antagonist could produce a paradoxical bronchoconstriction, attributable to concurrent antagonism of pre-junctional muscarinic auto-receptors, thereby reducing the effectiveness of post-junctional muscarinic M_3 receptor blockade (Morley, 1994). A muscarinic M_3 selective antagonist may therefore reduce bronchiolar constriction without augmentation of acetylcholine release (Loenders et al., 1992). However, the non-selective muscarinic antagonist ipotropium does not increase acetylcholine release in human trachea at concentrations expected to attain muscarinic M_3 receptor blockade (Patel et al., 1995). Interestingly, chronic exposure to non-selective muscarinic receptor antagonists up-regulates m2 and m3 mRNA, a finding that may explain the increase in bronchial hyperresponsiveness in animal models associated with continuous anticholinergic therapy (Witt-Enderby et al., 1995). Selective muscarinic M_1 receptor antagonists, such as telenzepine, may confer a protective mechanism against vagal overstimulation, although this was not seen in patients with nocturnal asthma (Cazzola et al., 1994) or COPD (Ukena et al., 1993).

In lieu of genuinely selective muscarinic M_3 receptor antagonists, some therapeutic approaches to selective blockade exploit differences in receptor kinetics or absorption. Tiotropium bromide (BA 679 BR), for example, is an antagonist with a preferential slow off-rate from muscarinic M_3 receptors with respect to muscarinic M_2 receptors (Haddad et al., 1994; Takahashi et al., 1994a). Clinical studies with this compound have shown it to be an effective agent in patients with COPD (Maesen et al., 1995). Ipratropium, alternatively, is a quaternized derivative of atropine and is poorly absorbed into the systemic circulation when given by inhalation (Lulich et al., 1995). Although non-selective between subtypes, the low systemic absorption of the antagonist facilitates selective antagonism of airway muscarinic receptors.

VI. Conclusions

This paper has reviewed the functional role(s) of muscarinic receptors in smooth muscle. The evolving data indicate that most smooth muscles contract in response to muscarinic M_3 receptor activation, even though this receptor forms a small percentage of the total muscarinic receptor population in the tissues studied to date. Nonetheless, under appropriate experimental conditions, the majority population of muscarinic M_2 receptor are functional, principally after depletion of muscarinic M_3 receptors. The parasympathetic control of smooth muscle contractility may thus occur directly, via the muscarinic M_3 receptor activation and indirectly, via M_2 receptor stimulation. In this model, the muscarinic M_2 receptor serves to oppose relaxant responses induced by elevation of intracellular cAMP. Consequently, the regulation of intracellular cAMP levels, as in the myocardium, is reciprocally regulated by the sympathetic and parasympathetic nervous systems. The physiological consequences of this model remain unknown, although one may speculate on its function. Muscle relaxation may occur during the relaxant phase of peristalsis, urinary bladder filling or pregnancy. This phase is presumably dominated by sympathetic drive to the muscle while parasympathetic control is inhibited. Alternatively, when active contraction takes place (during peristalsis or micturition, for example), both muscarinic M_2 and M_3 receptors are activated and the sympathetic system reciprocally inhibited.

Assuming that this model is physiologically appropriate, responses of smooth muscle to parasympathetic activation should be considered *equally* in terms of opposing relaxation and augmenting contraction. Participation of both muscarinic M_2 and M_3 receptors in the maintenance of muscle tone implies that current development of selective muscarinic M_3 receptor antagonists is at least arguable. Indeed, studies in isolated tissue, admittedly of animal origin, show that reducing the muscarinic M_3 receptor function predisposes the tissue to contraction via the muscarinic M_2 receptor. It is therefore conceivable that muscarinic M_2 and M_3 affinity may be desirable characteristics of novel anti-spasmolytics, assuming that tachycardia is not a side effect. It has not been reported whether the ratio of muscarinic M_2: M_3 receptors changes in diseases such as urge incontinence, irritable bowel syndrome, hypertension or glaucoma, although it does appear to change as a function of maturation. Interestingly, in a model of airway hypersensitivity, the muscarinic M_2 receptor may assume a greater importance, because in atopic sensitized rabbit trachea, the muscarinic M_2 receptor density and coupling are enhanced (Hakonarson et al., 1995). The paucity of information in this area clearly reflects the lack of data reported using human tissue from normal and diseased states.

It is now clear that the role of muscarinic receptor subtypes in smooth muscle function is both complex and subtle. Elucidating *how* complex and subtle will undoubtedly emerge from research data, preclinical and clinical, in future studies.

Acknowledgements. The authors thank Dr. R. D. Clark for preparing the structures shown in figure 2.

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