ELSEVIER

European Journal of Pharmacology 304 (1996) 211–219

ejp

Muscarinic receptors in developing rat colon [1]

Lubo Zhang *

Center for Perinatal Biology, Department of Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

Received 27 July 1995; revised 2 January 1996; accepted 9 February 1996

Abstract

Muscarinic receptor subtypes were characterized in fetal (21 day), newborn (3 day), and adult (3 month) rat colon smooth muscle. Saturation binding of the nonselective muscarinic antagonist radioligand [^{3}H]quinuclidinyl benzilate revealed a single class of binding sites in all three age groups. The binding affinities of [^{3}H]quinuclidinyl benzilate were not significantly different among three age groups (K_D: 0.19 → 0.27 nM). In contrast, the receptor densities (B_{max}, fmol/mg protein) showed a significant age-related decrease with fetus (518.9 ± 7.4) > newborn (480.3 ± 45.6) ≫ adult (192.4 ± 32.8). In both newborn and adult tissues, the muscarinic agonist carbachol bound to two sites with high and low affinities. Although the agonist binding affinities in the newborn tissue were not significantly different from those in the adult tissue, the high-affinity binding sites for carbachol were significantly increased in the later (41% → 61%). Addition of guanosine-5′-*O*-(3-thio)triphosphate (100 μM) abolished apparent high-affinity binding sites in both newborn and adult tissues. Antagonist competition binding in the newborn tissue indicated a homogeneous population of muscarinic M_2 receptors. Unlike in newborn tissues, the heterogeneous binding of pirenzepine and 4-diphenylacetoxy-*N*-methylpiperidine methobromide in adult tissues revealed coexistence of muscarinic M_3 (45%) and M_2 (55%) receptors. In accordance, activation of muscarinic receptors in the adult tissue stimulated synthesis of inositol 1,4,5-trisphosphate. These results suggest maturational changes of muscarinic receptor subtypes and their coupling to G proteins in rat colonic smooth muscle. These changes may account, at least in part, for developmental alterations of functional responses in colonic smooth muscle.

Keywords: Colon, rat; Development; Muscarinic receptor

1. Introduction

The parasympathetic neurotransmitter acetylcholine, acting postsynaptically at the smooth muscle muscarinic cholinergic receptors, is a principle determinant of colonic motility. While molecular cloning has revealed at least five distinct genes encoding different muscarinic receptors in human and rat (Bonner, 1989), four subtypes of the muscarinic receptor, M_1, M_2, M_3, and M_4 have been defined on the basis of the action of antagonists, either in radioligand binding or functional studies (Waelbroeck et al., 1990; Doods et al., 1994). In many tissues, it is common that disparate muscarinic receptor subtypes coexist and mediate distinct signal transduction pathways. We have demonstrated that in canine colonic smooth muscle M_2 and M_3 subtypes of muscarinic receptors coexist, and mediate inhibition of adenylyl cyclase and stimulation of phosphoinositide turnover through different G proteins (Zhang et al., 1991; Zhang and Buxton, 1991).

Although age-related changes in the muscarinic receptors and their coupling to G proteins have been the subject of several studies (Wills-Karp, 1993; Haxhiu-Poskurica et al., 1993; Whitsett and Hollonger, 1984; Blake et al., 1991; Anson et al., 1992), developmental aspects of muscarinic influences on colonic functions are not clear. The present study was undertaken to determine the binding characteristics of the nonselective muscarinic antagonist radioligand [^{3}H]quinuclidinyl benzilate in colonic smooth muscle of fetal, newborn, and adult rat. By examining the affinities of relatively selective muscarinic antagonists in competition of [^{3}H]quinuclidinyl benzilate binding, we evaluated the effects of animal development on the expression of muscarinic receptor subtypes. Because muscarinic receptors are coupled to G proteins (Zhang and Buxton, 1991; Wills-Karp, 1993; Haxhiu-Poskurica et al., 1993), which could be altered during development (Haxhiu-Poskurica et al., 1993; Wills-Karp, 1993; Anson et al., 1992), we also examined the effects of age on G protein

* Tel.: 909-824-4325; fax: 909-824-4029.

[1] Part of the results has been presented in Western Pharmacology Society 38th annual meeting, 1995.

PII S0014-2999(96)00130-6

coupling of muscarinic receptors by analyzing agonist competition binding. Our data suggest that expression of muscarinic M_2 receptors is down-regulated, whereas that of muscarinic M_3 receptors is up-regulated as a consequence of animal development. In addition, although agonist binding affinity is not altered, high-affinity guanine nucleotide-sensitive binding sites of the agonist are increased during development.

2. Materials and methods

2.1. Membrane preparation

Colons removed from fetal (21 day), newborn (3 day), and adult (3 month) Sprague-Dawley rats following decapitation or cervical dislocation were placed into ice-cold hypotonic buffer A (50 mM Tris base, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4). After washing out of fecal material, the mucosa was carefully removed. The smooth muscle was then minced with scissors, and homogenized in 10 volumes of the same buffer using a glass-glass homogenizer. The homogenate was filtered through a nylon cloth (500 μm), and centrifuged at 48 000 × g for 60 min at 4°C. The resulting supernatant was discarded, and the pellet was quickly frozen in liquid nitrogen and stored as a frozen powder at −80°C for up to 2 weeks until employed in radioligand binding studies.

2.2. Radioligand binding studies

Binding of the nonselective muscarinic receptor antagonist radioligand [^{3}H]quinuclidinyl benzilate was measured by a rapid filtration method similar to that described previously (Zhang et al., 1991). Membrane pellets, prepared as described above, were re-suspended in buffer A to yield approximately 0.4 mg/ml protein as determined by the method of Bradford (Bradford, 1976). Radioligand binding assays were carried out in triplicate in a 500 μl volume consisting of 440 μl of membrane suspension, 50 μl of radioligand, and 10 μl of drug or diluent. Saturation binding experiments employed concentrations of [^{3}H]quinuclidinyl benzilate from 0.02 to 5 nM. Nonspecific binding (about 10% at the K_D for [^{3}H]quinuclidinyl benzilate) was determined by the addition of 10 μM atropine. Equilibrium binding was carried out at 30°C for 75 min. Binding of radioligand was stable for 150 min and completely reversible. Bound and free [^{3}H]quinuclidinyl benzilate was separated by rapid filtration of the suspension over polyethylenimine (0.3%)-pre-treated filters (Whatman GF/C) using a Brandel cell harvester. Filters were rinsed with two 5-ml aliquot of ice-cold buffer B (5 mM Tris; 1 mM $MgCl_2$; 0.1 mM EGTA; pH 7.4) and counted for radioactivity at 45% efficiency in a Packard 1900CA Tri-Carb liquid scintillation counter (Packard Instrument Comp., Downers Grove, IL, USA).

Antagonist competition binding was performed by competition of binding of [^{3}H]quinuclidinyl benzilate in membranes prepared from colonic smooth muscle. Increased concentrations (1 nM to 0.1 mM) of 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (AF-DX 116), pirenzepine, and 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP) were employed to compete for binding of [^{3}H]quinuclidinyl benzilate. Total binding was determined in the absence of the competing antagonists, whereas nonspecific binding was determined in the presence of 10 μM atropine. Conditions of incubation and separation of bound from free [^{3}H]quinuclidinyl benzilate were the same as those in the saturation binding. Agonist binding was measured in the same design with increasing concentrations of carbachol (10 nM to 1 mM). Two characteristics of agonist binding were determined: agonist binding affinity and relative portions of high and low-affinity binding sites. Because agonist binding is regulated by G proteins, these experiments were performed in the absence and/or presence of guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) (100 μM), a stable GTP analog.

2.3. Measurement of tissue inositol phosphates

Tissue inositol phosphates were measured as described previously by us (Zhang and Buxton, 1991). Briefly, colons were dissected into 20 mg pieces, and incubated with *myo*-[^{3}H]inositol (200 μCi/ml) at 35°C for 3 h in oxygenated labelling buffer of the following composition (in mM): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 0.6; Na_2HPO_4, 0.6; $MgCl_2$, 1.2; dextrose, 5.0; $CaCl_2$, 0.5; Hepes, 10.0; and $NaHCO_3$, 5.0. As we have reported previously, at this concentration of the radiolabel, incorporation of *myo*-[^{3}H]inositol into the inositol containing phospholipids, phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate reached apparent steady state by 3 h in colonic smooth muscle (Zhang and Buxton, 1991). After labeling, tissues were washed with six changes of fresh Krebs' buffer over 30 min to remove unincorporated radioactivity. Following various treatments determined by experimental design, reactions were terminated by quick freezing in liquid nitrogen. Tissues were then homogenized in 1 ml ice-cold $CHCl_3$-CH_3OH-HCl (66:33:1) followed by addition of 2 ml H_2O. The homogenate was centrifuged at 400 × *g* for 10 min, and the aqueous phase collected and lyophilized to dryness. [^{3}H]Inositol phosphates were separated by high performance liquid chromatography over Whatman Partisil 5 SAX strong anion exchange column eluted with an ammonium phosphate gradient as described previously (Zhang and Buxton, 1991). The identities of the [^{3}H]inositol phosphates were verified by authentic standards obtained from DuPont-NEN (Boston, MA, USA). Quantification was achieved by determining peak area as recorded in counts per minute by a Radiomatic A500 Flow-one scintillation

counter (Packard Inst., Meridian, CA, USA) with ^3H efficiency of 52%.

2.4. Data analysis

Saturation binding data were analyzed by both Scatchard plot and computer-assisted nonlinear least-squares regression to determine dissociation constant (K_D) and receptor density (B_{max}) (GraphPAD Inplot version 4.03, GraphPAD Software, San Diego, CA, USA). Both methods yielded similar K_D and B_{max} values. For competition binding data, the nonlinear least-squares approach fits the data to either one or two classes of binding sites and assists in determining if the two-site model is significantly better than the one-site fit of the data (F-test). Results were expressed as means ± S.E., and the differences were evaluated for statistical significance ($P < 0.05$) by one-way analysis of variance.

2.5. Materials

The stable muscarinic agonist carbachol and the antagonists pirenzepine, atropine, 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP), and bovine serum albumin were purchased from Research Biochemicals International (Natick, MA). 11-2[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-6-one (AF-DX 116) was supplied by Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA). Guanosine-5′-*O*-(3-thio)triphosphate (GTPγS), ethylene glycol bis(ß-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and Tris base were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Quinuclidinyl benzilate (45.7 Ci/mmol), *myo*-[^{3}H]inositol (52.4 Ci/mmol) and authentic [^{3}H]inositol phosphate standards were obtained from DuPont-NEN (Boston, MA, USA).

3. Results

3.1. Saturation binding

Binding of [^{3}H]quinuclidinyl benzilate to membranes prepared from fetal, newborn, and adult rat colon smooth muscle was specific and saturable. Nonspecific binding represented about 10% of the total binding at concentrations of [^{3}H]quinuclidinyl benzilate near its dissociation constant (K_D, ~ 0.24 nM). In all three age groups, saturation isotherms using [^{3}H]quinuclidinyl benzilate were best described by an interaction of the radioligand with a single class of binding sites. As shown in Fig. 1, although K_D values for [^{3}H]quinuclidinyl benzilate in membranes of three age groups were not significantly different from each other (K_D: 0.19 → 0.27 nM, see Table 1), Scatchard analysis of [^{3}H]quinuclidinyl benzilate binding indicated a significant age-dependent decrease in muscarinic receptor density (B_{max}: 518.9 → 192.8 fmol/mg protein, $P < 0.01$, see Table 1).

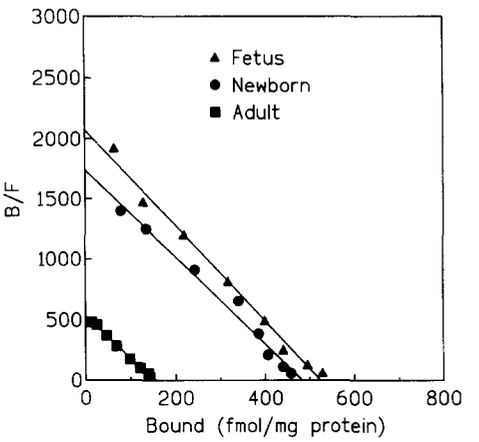

Fig. 1. Saturation binding of [^{3}H]quinuclidinyl benzilate (QNB) to fetal, newborn, and adult colon smooth muscle membranes. Tissue membranes were incubated at 30°C for 75 min with increasing concentrations of [^{3}H]QNB (0.02–5 nM). Specific [^{3}H]QNB binding was defined as the arithmetic difference between total binding and nonspecific binding observed in the presence of 10 μM atropine. The figure shows Scatchard plots representative of 3–7 independent experiments performed in triplicate for each age. ▲ fetus, ● newborn, ■ adult. Mean values of dissociation constant (K_D) and receptor density (B_{max}) are present in Table 1.

3.2. Agonist competition binding

One of the steps involved in age-related changes of receptor response is the alteration of receptor-G protein interaction evidenced by changes in high-affinity guanine nucleotide-sensitive agonist binding (Wills-Karp, 1993; Haxhiu-Poskurica et al., 1993). Therefore, the ability of the muscarinic agonist carbachol to compete for specific

Table 1
Characteristics of [^{3}H]quinuclidinyl benzilate saturation binding in fetal, newborn, and adult rat colon smooth muscle

	Dissociation constant (K_D) (nM)	Density (B_{max}) (fmol/mg protein)
Fetus	0.25 ± 0.02	518.9 ± 7.4
Newborn	0.19 ± 0.04	480.3 ± 45.6
Adult	0.27 ± 0.04	192.8 ± 32.8 [a]

Increasing concentrations (0.02 → 5 nM) of [^{3}H]quinuclidinyl benzilate were incubated with tissue membranes at 30°C for 75 min. Specific binding was defined as the arithmetic difference between total binding and nonspecific binding in the presence of 10 μM atropine. Scatchard plot analysis of the specific binding data confirmed that [^{3}H]quinuclidinyl benzilate bound to a single class of binding sites in all age groups. Data are means ± S.E. of 3–7 independent experiments performed in triplicate. One-way ANOVA analysis among three age groups indicated that dissociation constants (K_D) were not significantly different, but receptor densities (B_{max}) were significantly different ($P < 0.001$). Post-tests of Bonferroni t-tests indicated that difference in B_{max} between fetus and newborn was not significant ($P > 0.05$). [a] Values significantly different from those of fetus and newborn ($P < 0.01$).

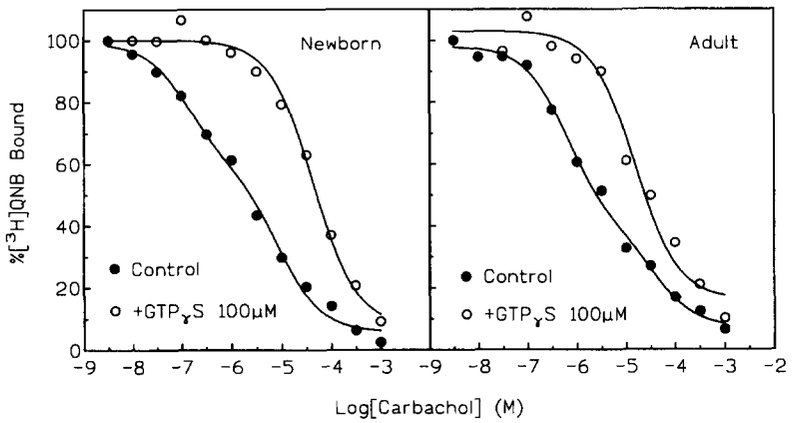

Fig. 2. Agonist competition of [^{3}H]quinuclidinyl benzilate (QNB) binding in newborn and adult colon smooth muscle membranes. Competition of [^{3}H]QNB (0.5 nM) binding by the muscarinic agonist carbachol was determined in the absence and presence of 100 μM GTPγS. Values, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 10 μM atropine (0%), are expressed as the percentage of maximal [^{3}H]QNB binding. Data were analyzed by nonlinear computer-based methods which yielded best-fits assuming two components of the agonist binding in the absence of GTPγS in both newborn and adult tissues. Data are representative of 4–5 independent experiments performed in triplicate for each age. ● control, ○ GTPγS 100 μM. Mean values of binding characteristics are present in Table 2.

[^{3}H]quinuclidinyl benzilate binding to muscarinic receptors in newborn and adult colon smooth muscle was studied in the absence and presence of the stable GTP analog GTPγS. As shown in Fig. 2, in the absence of GTPγS competition curves for carbachol were best fit by computer as a sum of binding to two classes of sites in both newborn and adult tissues. In the presence of 100 μM GTPγS, the competition curves for carbachol were steepened and shifted to the right, and the high-affinity binding sites for carbachol were abolished. The affinity of carbachol in the presence of GTPγS closely approximated the low-affinity component of the biphasic curve in the absence of GTPγS (Table 2). The results were consistent with a role for G proteins in coupling of the muscarinic receptors to responses in colon smooth muscle of both newborn and adult animals. In the absence of GTPγS, although neither high (K_{IH})- nor low (K_{IL})-affinity values for carbachol competition of [^{3}H]quinuclidinyl benzilate in newborn tissues were significantly different from those in adult tissues, the high-affinity binding sites for carbachol were significantly increased in the tissues from adult animals (41% → 61%, $P < 0.05$, see Table 2).

3.3. Antagonist competition binding

To examine the effect of development on muscarinic receptor subtypes in colonic smooth muscle, the relatively subtype-selective muscarinic antagonists AF-DX 116, pirenzepine, and 4-DAMP were used in radioligand binding competition studies with [^{3}H]quinuclidinyl benzilate. As shown in Fig. 3, all three antagonists competed for [^{3}H]quinuclidinyl benzilate binding in a concentration-dependent manner in both newborn and adult tissues. In newborn animals, analysis of competition curves indicated

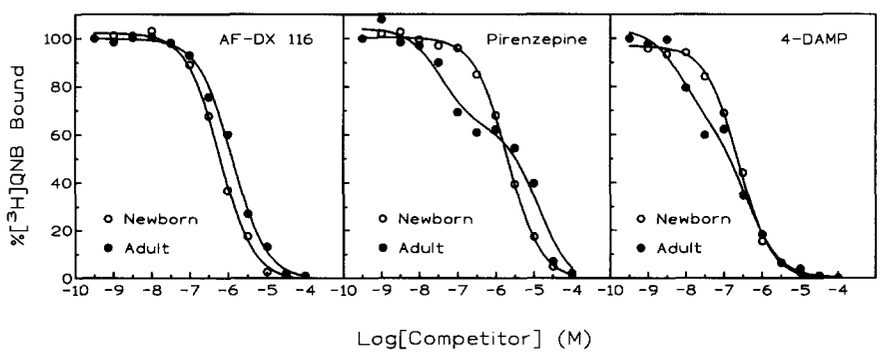

Fig. 3. Selective antagonist competition of [^{3}H]quinuclidinyl benzilate (QNB) binding in newborn and adult colon smooth muscle membranes. Tissue membranes were incubated with increasing concentrations of subtype-selective muscarinic antagonists AF-DX 116 (A), pirenzepine (B), and 4-DAMP (C) in the presence of [^{3}H]QNB (0.5 nM). Values, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 10 μM atropine (0%), are expressed as the percentage of maximal [^{3}H]QNB binding. Data were analyzed by nonlinear computer-based methods which yielded best-fits assuming two components of the binding for pirenzepine and 4-DAMP in the adult tissue. Data are representative of 4–6 independent experiments performed in triplicate for each age. ● adult, ○ newborn. Mean values of binding charactepistics are present in Table 3.

Table 2
Characteristics of the agonist binding to newborn and adult colon smooth muscle muscarinic receptors

	pK_{IH}	pK_{IL}	R_H(%)	R_L(%)
− GTPγS				
Newborn	7.11 ± 0.40	5.41 ± 0.31	41 ± 5.7	59 ± 5.7
Adult	6.77 ± 0.05	4.83 ± 0.14	61 ± 3.5 [a]	39 ± 3.5
+ GTPγS 100 μM				
Newborn		5.26 ± 0.17		100
Adult		5.21 ± 0.05		100

Muscarinic agonist carbachol affinities and proportions of high- and low-affinity sites (K_{IH}, K_{IL}, R_H, and R_L, respectively) in competition for [^{3}H]quinuclidinyl benzilate (0.3 nM) binding, in membranes prepared as described in Methods, were determined in the absence or presence of 100 μM GTPγS. In both newborn and adult tissues, addition of GTPγS abolished agonist high-affinity binding sites. Data are means ± S.E. of 4–5 independent experiments performed in triplicate. [a] Value significantly different from that of newborn ($P < 0.05$).

Table 3
Binding characteristics of selective muscarinic antagonists in competition of [^{3}H]quinuclidinyl benzilate binding in newborn and adult colon smooth muscle

	pK_{IH}	pK_{IL}	R_H	R_L	n_H
AF-DX 116					
Newborn		6.72 ± 0.02		100	1.01 ± 0.03
Adult		6.51 ± 0.13		100	0.95 ± 0.05
Pirenzepine					
Newborn		6.07 ± 0.08		100	0.97 ± 0.06
Adult	7.49 ± 0.02	5.69 ± 0.13	40 ± 3.6	60 ± 3.6	0.52 ± 0.04 [a]
4-DAMP					
Newborn		7.52 ± 0.23		100	1.03 ± 0.04
Adult	8.63 ± 0.13	7.32 ± 0.16	49 ± 7.7	51 ± 7.7	0.70 ± 0.05 [a]

Antagonist competition binding affinities and proportions of high- and low-affinity sites (K_{IH}, K_{IL}, R_H, and R_L, respectively) in competition for [^{3}H]quinuclidinyl benzilate (0.3 nM) binding, in membranes prepared as described in Methods, were determined. Data were analyzed by the nonlinear least-squares approach (GraphPAD Inplot) which fits the data to either one or two classes of binding sites and assists in determining if the two-site model is significantly better than the one-site fit of the data (F-test). Data are means ± S.E. of 4–6 independent experiments performed in triplicate. [a] Values statistically different from unity ($P < 0.01$).

a single component binding of all three antagonists. The rank order of the inhibition constant (K_I) values of muscarinic receptors in newborn colon for the antagonists used was 4-DAMP > AF-DX 116 > pirenzepine (see Table 3). The same rank order of potency has been obtained for these antagonists in binding to muscarinic M_2 receptors in a number of other tissues and cell types (Buckley et al., 1989; Doods et al., 1987; Hulme et al., 1990; Zhang et al., 1991), indicating a homogeneous population of the muscarinic M_2 receptors in newborn rat colon.

In agreement with newborn tissues, analysis of AF-DX 116 competition curves in adult tissues indicated a single component binding of this antagonist with similar inhibition constant as that determined in newborn tissues (Fig. 3 and Table 3). However, unlike in newborn tissues, analysis of competition curves with pirenzepine and 4-DAMP in adult tissues indicated that they interact with more than one class of binding sites (Fig. 3). Curves were best fit by a sum of interaction with two classes of binding sites for each antagonist (Table 3). The percentage of high-affinity binding sites of pirenzepine was not significantly different from that of 4-DAMP. Further, the low affinities of both pirenzepine and 4-DAMP determined in adult tissues were not significantly different from those obtained in newborn tissues. These results suggest that, aside from the M_2 subtype, another subtype of muscarinic receptor in colon smooth muscle has been expressed during development.

The appearance of high-affinity binding sites for 4-DAMP and pirenzepine in adult tissues raised the possibility of existence of muscarinic M_3 receptors (Zhang et al., 1991). To further characterize the muscarinic receptor subtypes in adult colon smooth muscle, we examined the relative affinities of these antagonists in competition with [^{3}H]quinuclidinyl benzilate for binding in the heart and

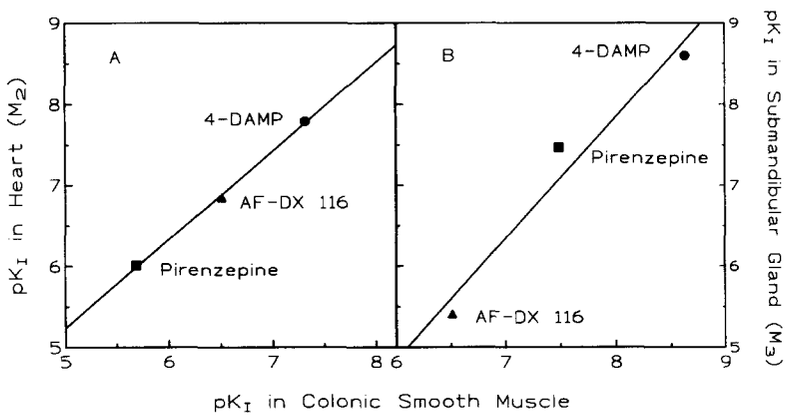

Fig. 4. Correlation between antagonist inhibition constants in adult colon smooth muscle and those in heart and submandibular gland. Panel A shows the correlation between subtype-selective muscarinic antagonist inhibition constants (pK_I) with low affinities of pirenzepine and 4-DAMP in colon smooth muscle showed in Table 3 and those in heart ($r^2 = 0.99$). Panel B shows the correlation between subtype-selective muscarinic antagonist inhibition constants (pK_I) with high affinities of pirenzepine and 4-DAMP in colon smooth muscle showed in Table 3 and those in submandibular gland ($r^2 = 0.97$). ▲ AF-DX 116, ■ pirenzepine, ● 4-DAMP.

submandibular gland, which exhibit a homogeneous population of muscarinic M_2 and M_3 receptors, respectively (Doods et al., 1987). In both of these tissues, the competition curves for pirenzepine and 4-DAMP were best fit assuming the presence of a single class of binding sites for each antagonist (data not shown). As shown in Fig. 4, the antagonist affinities (with high affinity of pirenzepine and 4-DAMP) determined in adult colon smooth muscle were highly correlated to those in the submandibular gland (muscarinic M_3 receptors, $r^2 = 0.97$), whereas the antagonist affinities (with low affinity of pirenzepine and 4-DAMP) in colon were highly correlated to those determined in the heart (muscarinic M_2 receptors, $r^2 = 0.99$). These results suggest that both muscarinic M_3 and M_2 receptors coexist in adult colon.

Because the kinetic of [^{3}H]quinuclidinyl benzilate binding to muscarinic M_3 receptors is relatively slow, we examined [^{3}H]quinuclidinyl benzilate binding in rat submandibular gland which exhibits a homogeneous population of muscarinic M_3 receptors. Under the same conditions used in colon tissues, [^{3}H]quinuclidinyl benzilate bound to a large numder of muscarinic M_3 receptors in the submandibular gland ($B_{max} = 518.3 \pm 109.2$ fmol/mg protein) with the affinity ($K_D = 0.30 \pm 0.04$ nM) not different from that observed in the colon. The result suggests that [^{3}H]quinuclidinyl benzilate is able to label a significant proportion of muscarinic M_3 receptors in the colon in the present study.

3.4. Phosphoinositide hydrolysis

To further support the idea that muscarinic M_3 receptors, but not muscarinic M_4 receptors were expressed in addition to muscarinic M_2 receptors in adult tissues, we examined phosphoinositide hydrolysis in response to muscarinic agonist carbachol in adult colon smooth muscle. It is well known that muscarinic M_3 receptors are efficiently coupled to phosphoinositide hydrolysis (Peralta et al., 1988; Lechleiter et al., 1990, 1991; Ashkenazi et al., 1989; Zhang and Buxton, 1991). In the absence of the agonist, there was a significant inositol 1,4,5-trisphosphate signal, suggesting a basal phosphoinositide turnover activity in the tissue. As shown in Table 4, the generation of [^{3}H]inositol 1,4,5-trisphosphate was increased 54% in the presence of carbachol (100 μM, 30 min), a result that was reflected in the 400% increase seen in total [^{3}H]inositol phosphates accumulation under these conditions. Accumulation of both [^{3}H]inositol 1,4,5-trisphosphate and total [^{3}H]inositol phosphates following carbachol stimulation was effectively blocked by 1 μM atropine, confirming muscarinic receptor-mediated responses.

Table 4
Stimulation of inositol phosphate accumulation by carbachol in adult rat colon smooth muscle

	Ins(1,4,5)P_3	Total InsPs
Basal	82.6 ± 2.1	843.2 ± 33.6
Carbachol	127.5 ± 7.5 [a]	4514.6 ± 322.4 [a]
Carbachol + atropine	84.9 ± 13.9	979.4 ± 145.2

myo-[^{3}H]Inositol-prelabeled tissues were incubated, in the presence of 10 mM LiCl, with 100 μM carbachol for 30 min. Where appropriate, atropine (1 μM) was incubated with tissues for 15 min before the addition of carbachol. Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and total inositol phosphates (InsPs) were extracted and separated by high performance liquid chromatography, as described in the text. Data are means ± S.E. of 3 experiments. [a] Values (cpm/mg of tissue wet weight) statistically different from basal values ($P < 0.01$).

4. Discussion

The affinities of [^{3}H]quinuclidinyl benzilate in the present study were in close agreement with those determined for muscarinic receptors in canine colon smooth muscle and a number of other tissues and cell types (Zhang et al., 1991; Lucchesi et al., 1990; Wills-Karp, 1993; Mattera et al., 1985). While the binding of [^{3}H]quinuclidinyl benzilate to cholinergic nerve terminals could not be excluded, the method we used to prepare colonic smooth muscles minimized the influence of the nerve on the binding studies (Zhang et al., 1991, 1992). The finding of no age-related change in the affinity of [^{3}H]quinuclidinyl benzilate in this study suggests that the ability of the receptors to bind quinuclidinyl benzilate remains stable with age. This observation is consistent with previous reports in a variety of tissues (Wills-Karp, 1993; Blake et al., 1991; Whitsett and Hollonger, 1984; Haxhiu-Poskurica et al., 1993). The decline in muscarinic receptor density with age determined by [^{3}H]quinuclidinyl benzilate binding suggests that the alteration in developmental aspects of muscarinic influences on colonic functions may occur at some point during protein synthesis of the receptors or in the availability of the receptors to bind the ligand. Similar results have been obtained from rat cerebral cortex (Van-Huizen et al., 1994), cochlea (Bartolami et al., 1993), and tracheal smooth muscle (Whitsett and Hollonger, 1984), where muscarinic receptor density is higher in newborn tissues than adult tissues. Although the mechanism(s) underlying the development-related decline of muscarinic receptors in colonic smooth muscle is not clear at present, it may be due, at least in part, to the immaturity of parasympathetic innervation in newborn animals (Yagi et al., 1991; Haxhiu-Poskurica et al., 1993). With the maturing of the nerve innervation during postnatal period, increasing release of acetylcholine could down-regulate the muscarinic receptors in the smooth muscle.

Competition of specific [^{3}H]quinuclidinyl benzilate binding by a series of muscarinic antagonists satisfied the criteria of the muscarinic M_2 receptors in the newborn tissues. Although the absolute affinity of a drug for its receptor may vary according to assay conditions and from tissue to tissue, the relative potency of different drugs

towards the same receptor is less variable and, thus, presents a more consistent criterion for comparison. The rank order of potencies for the antagonists used in newborn tissues was 4-DAMP > AF-DX 116 > pirenzepine. The same rank order of the antagonists has been reported for muscarinic M_2 receptors in both binding and functional studies (Zhang et al., 1991; Buckley et al., 1989; Doods et al., 1987; Hulme et al., 1990). Whereas 4-DAMP are generally found to be more potent than AF-DX 116 and pirenzepine for binding to either muscarinic M_2 or M_3 receptors, AF-DX 116 is consistently seen as more potent than pirenzepine for binding to the muscarinic M_2 receptor in all tissues and cell types thus far examined. In contrast, pirenzepine is more potent than AF-DX 116 in binding to muscarinic M_3 receptors. The presence of muscarinic M_2 receptors in the newborn colon was further supported by the finding that there was an excellent correlation between the antagonist affinities determined in newborn colon and those in the heart, in which a homogeneous population of muscarinic M_2 receptors exists. The absence of heterogeneous binding of the antagonists examined in the present study suggests that a homogeneous population of muscarinic M_2 receptors exists in the newborn colon.

The finding that pirenzepine and 4-DAMP bound to two components of binding sites in adult colon suggests that, aside from muscarinic M_2 receptors, another subtype has been expressed in rat colon during development. The appearance of binding sites with high affinity for 4-DAMP, which is consistent with those reported for muscarinic M_3 receptors, in adult tissues suggests that the development-related subtype is muscarinic M_3 receptors. Further, we have previously demonstrated that pirenzepine distinguishes muscarinic M_3 and M_2 receptors in canine colonic smooth muscle with high affinity for the M_3 subtype and low affinity for the M_2 subtype (Zhang et al., 1991). The finding that the binding curve of AF-DX 116 was monophasic is distinct from data reported in rat colon (Gomez et al., 1992), but is in agreement with that in canine colon (Zhang et al., 1991). The differences are not clear at present. Although many studies have demonstrated a 20–35-fold difference in the affinities of the cardiac and glandular muscarinic receptors for AF-DX 116 (Hammer et al., 1986; Giraldo et al., 1987; Martos et al., 1987), less selectivity has also been reported (Buckley et al., 1989; Michel and Whiting, 1988; Zhang et al., 1991).

While the high-affinity binding sites of 4-DAMP and pirenzepine in the adult tissue could also be in accordance with the presence of muscarinic M_4 receptors, the results of the present study argue against this possibility for three reasons. First, although the binding affinities may vary, the potency ratio of pirenzepine and AF-DX 116 in binding to any specific subtype of muscarinic receptors is remarkably consistent in many studies (Doods et al., 1987, 1994; Waelbroeck et al., 1990). Pirenzepine has been found 10- and 3-fold more potent than AF-DX 116 in binding to muscarinic M_3 and M_4 receptors, respectively. The present finding of a 10-fold difference between pirenzepine high affinity and the affinity of AF-DX 116 (see Table 3) suggests that pirenzepine high-affinity binding sites are muscarinic M_3, but not M_4, receptors. Second, it is well documented that muscarinic M_3 receptors are efficiently coupled to phosphoinositide hydrolysis, while muscarinic M_4 receptors are coupled to adenylyl cyclase. In the present study, we have demonstrated that activation of muscarinic receptors in adult rat colon stimulates phosphoinositide hydrolysis, consisting with the presence of muscarinic M_3 receptors. Finally, the high affinities of 4-DAMP and pirenzepine determined in the colon are the same as those determined in the submandibular gland (muscarinic M_3 receptors). The finding that the affinity of AF-DX 116 determined in the colon was not different from that in the heart, but is 10-fold higher than that in the submandibular gland is consistent with the presence of muscarinic M_2 receptors (55%) in adult rat colon (Buckley et al., 1989; Doods et al., 1987; Hulme et al., 1990). Taken together, these results provide strong evidence that both muscarinic M_3 and M_2 receptors coexist in adult rat colon. The coexistence of muscarinic M_2 and M_3 receptors in the smooth muscle has also been demonstrated in ileum (Candell et al., 1990), trachea (Lucchesi et al., 1990), stomach (Hearwi et al., 1988), coronary artery (Entzeroth et al., 1990), and canine colon (Zhang et al., 1991). The proportion of binding sites with high affinity for 4-DAMP (49%) was not significantly different from that for pirenzepine (40%), suggesting that an average of 45% of the receptor sites are muscarinic M_3 receptors.

It is clear from the present study that the expression of muscarinic receptor subtypes in colon smooth muscle is under different regulations during development (see Fig. 5). The functional significance of these maturational changes is not entirely clear. It has been found that con-

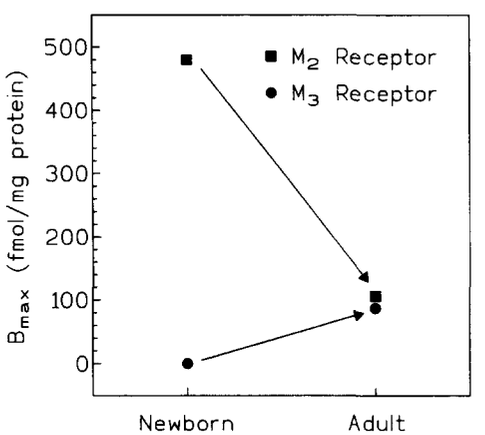

Fig. 5. Effect of development on the density of muscarinic M_2 and M_3 receptors in colon smooth muscle. The density (B_{max}) of muscarinic M_2 (■) and M_3 (●) receptors in newborn and adult colon smooth muscle was calculated based on [^{3}H]QNB saturation binding and the finding of 100% muscarinic M_2 receptors in newborn tissues and 45% muscarinic M_3 and 55% muscarinic M_2 receptors in adult tissues.

tractions of colonic smooth muscle to muscarinic agonists are significantly increased in adult compared to newborn animals (Yagi et al., 1991). In our previous study with canine colonic smooth muscle, we have demonstrated that the relative density of muscarinic M_3 receptors is higher on the cell surface than the cell interior (Zhang and Buxton, 1993). That most of the M_3 binding sites were found on the cell surface suggests the relative importance of muscarinic M_3 receptor signalling in contractile responses to acetylcholine in vivo. Whereas it is not intellectually clear of the role played by muscarinic M_2 receptor-mediated lowering of cAMP production in the muscarinic-induced contraction of the tissue, activation of muscarinic M_3 receptors stimulates generation of inositol 1,4,5-trisphosphate which releases Ca^{2+} from intracellular stores and produces contractions of the smooth muscle. The finding that antagonism of the muscarinic-induced contraction by muscarinic receptor subtype-selective antagonists revealed involvement of muscarinic M_3 receptors in a variety of tissues indicates an important role for muscarinic M_3 receptors in the regulation of smooth muscle contractions.

Not only were muscarinic receptor subtypes in rat colon altered during development, the coupling between muscarinic receptors and G proteins was also changed. In agreement with the finding in canine colon (Zhang et al., 1991), the agonist competition curve in the present study exhibited the biphasic nature in both newborn and adult tissues, which likely reflected the state of interaction of muscarinic receptors with endogenous G proteins. This was confirmed by addition of 100 μM nonhydrolyzable GTP analogue GTPγS, which abolished the agonist high-affinity binding and produced a proportional increase in the number of receptors with low affinity for the agonist. In the absence of GTPγS, although neither high (K_{IH})- nor low (K_{IL})-affinity values for carbachol in competition of [^{3}H]quinuclidinyl benzilate in newborn animals were significantly different from those in adult animals, the high-affinity binding sites for carbachol were significantly increased in the tissues from adult animals (41% → 61%) (see Table 2). Because the total muscarinic receptor number was decreased from newborn to adult, the high-affinity binding sites for the agonist was, in fact, decreased from 197 to 117 fmol/mg protein in adult as compared to newborn rat colon. However, the decrease in the receptor density (60%) was over that in the high-affinity binding sites (40%). The results suggest that muscarinic receptors may be in excess over available G proteins in newborn rats but less so in adult rats, and suggest an enhancement of the coupling efficiency of G proteins with the receptors in adult rats. Similar results have been found in the piglet tracheal smooth muscle in which high-affinity muscarinic receptors coupled to G proteins were not detectable in < 7-day-old piglets and fully developed in adult animals (Haxhiu-Poskurica et al., 1993). Because coupling of muscarinic receptors with G proteins is required for physiological activity, the maturation of the coupling may play one of the major roles in the regulation of the level of a given physiological response such as the contraction of the colonic smooth muscle.

In summary, these data have demonstrated the maturational changes of muscarinic receptor subtypes and their coupling to G proteins in rat colon smooth muscle. There was a significant development-related decrease in muscarinic M_2 receptors. In contrast, muscarinic M_3 receptors were up-regulated as a function of development. In addition, the coupling efficiency of G proteins to muscarinic receptors were enhanced during development. These maturational changes of muscarinic receptors may account, at least in part, for developmental changes of functional responses in colonic smooth muscle.

Acknowledgements

This work was supported by the intramural grant from Loma Linda University School of Medicine. The author thanks Mr Jason H.S. Chang for his technical assistance, Dr Lawrence D. Longo for providing tissues, and Dr Iain L.O. Buxton for the gift of AF-DX 116.

References

Anson, R.M., R. Cutler, J.A. Joseph, K. Yamagami and G.S. Roth, 1992, The effects of aging on muscarinic receptor/G-protein coupling in the rat hippocampus and striatum, Brain Res. 598, 302.

Ashkenazi, A., E. Peralta, J. Winslow, J. Ramachandran and D. Capon, 1989, Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell, Cell 56, 487.

Bartolami, S., M. Planche and R. Pujol, 1993, Characterization of muscarinic binding sites in the adult and developing rat cochlea, Neurochem. Int. 23, 419.

Blake, M.J., N.M. Appel, J.A. Joseph, C.A. Stagg, M. Anson, E.B. de Souza and G.S. Roth, 1991, Muscarinic acetylcholine receptor subtype mRNA expression and ligand binding in the aged rat forebrain, Neurobiol. Aging 12, 193.

Bonner, T.I., 1989, The molecular basis of muscarinic receptor diversity, Trends Neurosci. 12, 148.

Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Buckley, N.J., T.I. Bonner, C.M. Buckley and M.R. Brann, 1989, Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells, Mol. Pharmacol. 35, 469.

Candell, L.M., S.H. Yun, L.L.P. Tran and F.J. Ehlert, 1990, Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum, Mol. Pharmacol. 38, 689.

Doods, H.N., M.J. Mathy, D. Davidesko, K.J. Van Charldorp, A. De Jonge and P.A. Van Zwieten, 1987, Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors, J. Pharmacol. Exp. Ther. 242, 257.

Doods, H.N., M. Entzeroth, H. Ziegler, N. Mayer and P. Holzer, 1994, Pharmacological profile of selective muscarinic receptor antagonisits on guinea-pig ileal smooth muscle, Eur. J. Pharmacol. 253, 275.

Entzeroth, M., H.N. Doods and N. Mayer, 1990, Characterization of

porcine coronary muscarinic receptors, Naunyn-Schmied. Arch. Pharmacol. 341, 432.

Giraldo, E., E. Monteferini, H. Ladinsky and R. Hammer, 1987, Muscarinic receptor heterogeneity in guinea pig intestinal smooth muscle: binding studies with AF-DX 116, Eur. J. Pharmacol. 141, 475.

Gomez, A., F. Martos, I. Bellido, E. Marquez, A.J. Garcia, J. Pavia and F. Sanchez de la Cuesta, 1992, Muscarinic receptor subtypes in human and rat colon smooth muscle, Biochem. Pharmacol. 43, 2413.

Hammer, R., E. Giraldo, G.B. Schiavi, E. Monteferini and H. Ladnisky, 1986, Binding profile of a novel cardioselective muscarinic receptor antagonist AF-DX 116 to membranes of peripheral tissues and brain in the rat, Life Sci. 38, 1653.

Haxhiu-Poskurica, B., P. Ernsberger, M.A. Haxhiu, M.J. Miller, L. Cattarossi and R.J. Martin, 1993, Development of cholinergic innervation and muscarinic receptor subtypes in piglet trachea, Am. J. Physiol. 264, L606.

Hearwi, M., G. Lambrecht, E. Mutschler, U. Moser and A. Pfeiffer, 1988, Different binding properties of muscarinic M_2-receptor subtypes for agonists and antagonists in porcine gastric smooth muscle and mucosa, Gastroenterology 94, 630.

Hulme, E.C., N.J.M. Birdsall and N.J. Buckley, 1990, Muscarinic receptor subtypes, Annu. Rev. Pharmacol. Toxicol. 30, 633.

Lechleiter, J., R. Hellmiss, K. Duerson, D. Ennulat, N. David, D. Clapham and E. Peralta, 1990, Distinct sequence elements control the specificity of G protein activation by muscarinic acetylcholine receptor subtypes, EMBO J. 9, 4381.

Lechleiter, J., S. Girard, D. Clapham and E. Peralta, 1991, Subcellular patterns of calcium release determined by G protein-specific residues of muscarinic receptors, Nature 350, 505.

Lucchesi, P.A., C.R. Scheid, F.D. Romano, M.E. Kargacin, D. Mullikin-Kilpatrick, H. Yamaguchi and T.W. Honeyman, 1990, Ligand binding and G protein coupling of muscurinic receptors in airway smooth muscle, Am. J. Physiol. 258, C730.

Martos, F., E. Monteferini, E. Giraldo, A.M. De Paoli and R. Hammer, 1987, Characterization of muscarinic receptors in salivary and lacrimal glands of the rat, Eur. J. Pharmacol. 143, 189.

Mattera, R., B.J.R. Pitts, M.L. Entman and L. Birnbaumer, 1985, Guanine nucleotide regulation of a mammalian myocardial muscarinic receptor system, J. Biol. Chem. 260, 7410.

Michel, A.D. and R.L. Whiting, 1988, Methoctramine reveals heterogeneity of M_2 muscarinic receptors in longitudinal ileal smooth muscle membranes, Eur. J. Pharmacol. 145, 305.

Peralta, E.G., A. Ashkenazi, J.W. Winslow, J. Ramachandran and D.J. Capon, 1988, Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes, Nature 334, 434.

Van-Huizen, F., D. March, M.S. Cynader and C. Shaw, 1994, Muscarinic receptor characteristics and regulation in rat cerebral cortex: changes during development, aging and the oestrous cycle, Eur. J. Neurosci. 6, 237.

Waelbroeck, M., M. Tastenoy, J. Camus and J. Christophe, 1990, Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain, Mol. Pharmacol. 38, 267.

Whitsett, J.A. and B. Hollonger, 1984, Muscarinic cholinergic receptors in developing rat lung, Pediatr. Res. 18, 1136.

Wills-Karp, M., 1993, Age-related changes in pulmonary muscarinic receptor binding properties, Am. J. Physiol. 265, L103.

Yagi, H., W.J. Snape, Jr. and P.E. Hyman, 1991, Developmental changes in agonist-mediated colonic smooth muscle contraction in the rabbit, Pediatr. Res. 29, 20.

Zhang, L. and I.L.O. Buxton, 1991, Musacrinic receptors in canine colonic circular smooth muscle II. Signal transduction pathways coupled to the muscarinic receptors, Mol. Pharmacol. 40, 952.

Zhang, L. and I.L.O. Buxton, 1993, Protein kinase regulation of muscarinic receptor signalling in colonic smooth muscle, Br. J. Pharmacol. 108, 613.

Zhang, L., B. Horowitz and I.L.O. Buxton, 1991, Muscarinic receptors in canine colonic circular smooth muscle I. Coexistence of M_2 and M_3 subtypes, Mol. Pharmacol. 40, 943.

Zhang, L., K.D. Keef, M.E. Bradley and I.L.O. Buxton, 1992, Action of α_{2A}-adrenergic receptors in circular smooth muscle of canine proximal colon, Am. J. Physiol. 262, G517.