0026-895X/88/060617-09$02.00/0

MOLECULAR PHARMACOLOGY, 33:617-625

Characterization of Muscarinic Receptors in Guinea Pig Ileum Longitudinal Smooth Muscle

ETTORE GIRALDO, MARIA ALESSANDRA VIGANÒ, RUDOLF HAMMER, and HERBERT LADINSKY

Department of Biochemistry, Istituto De Angeli S.p.A., 20139 Milan, Italy (E.G., M.A.V., H.L.), and FL Biochemistry, Boehringer Ingelheim Zentrale GmbH, 6507 Ingelheim am Rhein, Federal Republic of Germany (R.H.)

Received November 17, 1987; Accepted March 21, 1988

SUMMARY

Heterogeneity in the muscarinic receptor population of guinea pig ileum longitudinal smooth muscle was found in competition binding experiments against *N*-methyl[^{3}H]scopolamine using either a cardioselective (AF-DX 116) or a smooth muscle-selective (hexahydrosiladifenidol) antimuscarinic compound. AF-DX 116 recognized 65% of the total receptors with high affinity and 35% with low affinity. Hexahydrosiladifenidol distinguished 24% of the total receptors with high affinity and 76% with low affinity. The two affinity binding constants displayed in smooth muscle by the compounds were similar to those of heart and glands, suggesting that the muscarinic receptor population in the smooth muscle is formed of about 30% glandular type and 70% cardiac type of the M_2 receptors. In dissociation experiments, the rate of breakdown of the *N*-methyl[^{3}H]scopolamine receptor complex in the smooth muscle was rapid and similar to the dissociation of *N*-methyl[^{3}H]scopolamine from muscarinic receptors in cardiac membranes, supporting the evidence for the presence of a large fraction of the cardiac receptor type in smooth muscle. To further characterize the population of the smooth muscle receptors recognized as glandular type, we performed protection experiments with hexahydrosiladifenidol, which binds to glandular M_3 receptors with high affinity. Smooth muscle membranes were initially incubated with this compound and then phenoxybenzamine was added to irreversibly alkylate the remaining unprotected receptors. Data from competition and dissociation binding experiments showed that, under these conditions, this protected fraction of the total receptor population in ileum smooth muscle had all the characteristics of the glandular type, i.e., slow *N*-methyl[^{3}H]scopolamine dissociation and affinity constants for a series of selective and nonselective muscarinic antagonists in the same order of magnitude as those found in the glandular tissue. These findings, together with the known observation that hexahydrosiladifenidol is more potent in inhibiting the functional activation of muscarinic receptors in smooth muscle relative to heart, lead to the hypothesis that smooth muscle contractility is mediated by a muscarinic receptor subtype similar to that found in glandular tissue.

The recent discovery of a novel selective antimuscarinic compound, AF-DX 116 (1), has provided deeper insight into the previous classification of the mAChRs (2–9). The M_2 type, as formerly defined with PZ, could be further divided into two different subclasses based on *in vitro* and *in vivo* data from AF-DX 116 studies, a cardiac M_2 subtype, which displays high affinity for AF-DX 116, and a glandular M_3 subtype, which has low affinity for the drug. Summarizing the available pharmacological data, the muscarinic system seems to be formed of three main mAChR subclasses, M_1 (in neuronal tissues such as brain, enteric, and sympathetic ganglia), M_2 (mainly in heart but also in some brain regions), and M_3 (essentially in exocrine glands and even in different brain regions) (10–22).

The existence of different mAChR subtypes has been recently shown also by various groups using the receptor cloning technique. The porcine cerebral (M_1) and cardiac (M_2) receptors were characterized and differentiated on the basis of their primary structures (23, 24). Moreover, analysis of genomic clones has revealed that there are at least four functional mAChR genes present in the brain of humans and rats (25).

The characterization of the mAChRs in smooth muscle has been less well clarified. With the use of PZ (26–29), it was established that smooth muscle contains an M_2 receptor type, but whether this M_2 is a cardiac or a glandular receptor, a combination of both, or yet another type could not be differentiated by the compound used. Consequently, it is not possible to categorize the mAChRs of smooth muscle within the M_2 subclass with any certainty. However, emerging evidence indicates that, functionally, the mAChRs present in ileum smooth muscle and atria may be considered to be different (11, 16, 28, 30, 31).

In preliminary binding experiments we found that, whereas

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; [^{3}H]NMS, *N*-methyl[^{3}H]scopolamine methyl chloride; NMS, *N*-methylscopolamine; ATR, atropine sulfate; QNB, 3-quinuclidinyl benzilate, racemic mixture; AF-DX 116, (11-[[[2-(diethylamino) methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepine-6-one); PZ, pirenzepine; HHSiD, hexahydrosiladifenidol; PBZ, phenoxybenzamine; 4-DAMP, 4-diphenylacetoxy *N*-methyl piperidine methiodide; CPPS, cyclohexylphenyl 2-piperidinoethyl silanol.

PZ recognized a uniform mAChR population in smooth muscle with a low affinity (600–1000 nM),[1] AF-DX 116 brought out a particular behavior in a number of smooth muscles of the gastrointestinal tract (32, 33), urinary bladder (34), and respiratory tract (17), indicating receptor heterogeneity in each preparation. Moreover, it was demonstrated that in isolated organs AF-DX 116, differently from PZ, had the ability to discriminate between atrial and ileal mAChRs (11, 16).

On the other hand, another antimuscarinic compound, HHSiD, showed inverse pharmacological selectivity with respect to AF-DX 116 in isolated organ preparations (35, 36), displaying greater potency in inhibiting smooth muscle contraction. In this study, we exploited the antithetic selectivity properties of AF-DX 116 and HHSiD to characterize the mAChR population of smooth muscle. Competition and dissociation binding experiments were performed on membranes from guinea pig ileum longitudinal smooth muscle after protecting one of the subtypes with HHSiD (or AF-DX 116) and then irreversibly alkylating the other with PBZ (37).

Materials and Methods

Tissue Preparation

Homogenization procedure. Male guinea pigs (Dunkin Hartley, 400–500 g) were used. Ileum longitudinal smooth muscle was removed by slipping ileal segments (about 10 cm long) rinsed with cold Tyrode's buffer, pH 7.5, over a tapered glass rod and then shearing away the longitudinal muscle with a buffer-wetted cotton swab (38). The muscle tissue was weighed and homogenized for 30 sec at maximal speed with an Ultra-Turrax in ice-cold Tyrode's buffer, pH 7.5, and then suspended in a glass-Teflon Potter-Elvehjem homogenizer (15 strokes), diluted 1/50 (w:v), and filtered through two layers of cheesecloth.

Heart and submandibular salivary glands were removed, cleansed, weighed, homogenized in ice-cold Tyrode's buffer, pH 7.5, and diluted 1/50 (w:v), following the previously described procedure. For other experiments, an artificial tissue was prepared by combining cardiac membranes (1/50; w:v) with glandular membranes (1/50; w:v) in a proportion of 70:30.

Receptor protection. A scheme depicting the various steps of drug addition, incubation, centrifugation, and subsequent washing used in the protection experiments is shown in Fig. 1. Aliquots (15 ml) of the ileum longitudinal smooth muscle homogenates were incubated with either 3 μM HHSiD or 10 μM AF-DX 116 for 30 min at 30°. This was followed by the addition of 100 μM PBZ and the homogenates were incubated for another 30 min. Pilot experiments were performed at different concentrations of AF-DX 116 (10 nM to 10 μM), HHSiD (10 nM to 10 μM), and PBZ (10 nM to 100 μM) and incubation times of 10–30 min. The conditions of drug concentrations and exposure times to the drugs were selected to achieve reproducible alkylation and to yield ≤10% (5.87 ± 0.82, mean ± SE, 19 experiments) of total number of receptors, ensuring the presence of a homogeneous receptor population sufficient to perform binding studies. After incubation was terminated, the homogenate was centrifuged at 20,000 × *g* for 20 min at 4°, the supernatant was discarded, and the pellet was washed with Tyrode's buffer and resuspended by continuous gentle aspirations with a syringe to avoid foaming. Centrifugations and resuspensions in 10 ml of Tyrode's buffer were performed twice more. This ensured the elimination of all unbound (reversible plus irreversible) and bound (reversible) ligand; further washing did not modify binding results. The final pellet was transferred to a glass-Teflon Potter-Elvehjem homogenizer, gently homogenized, and diluted.

Homogenates of glands or the artificial mixture of cardiac and glandular membranes were treated as above. Aliquots of control homogenates of the different tissues were prepared in parallel, following the same procedure but omitting the drugs. For the heart, only the control procedure was followed.

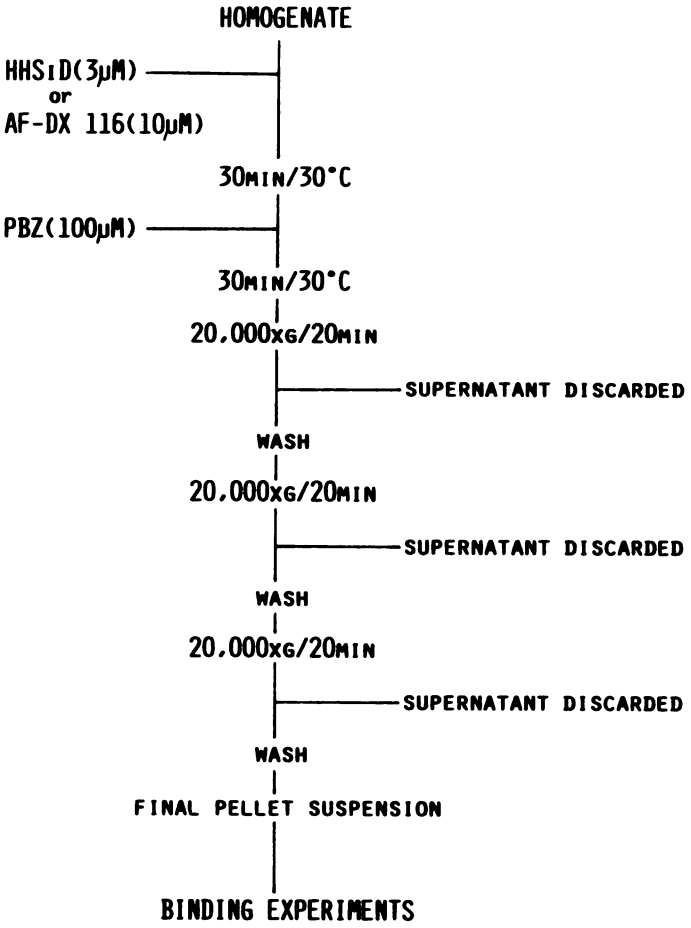

Fig. 1. Flow chart of the procedure used to selectively protect the glandular or the cardiac muscarinic receptor subtype from the alkylating agent PBZ.

Tissue dilution. For binding experiments, membrane suspensions were filtered through one layer of cheesecloth and diluted with ice-cold Tyrode's buffer, pH 7.5, to yield a final protein concentration (μg/ml) for each tissue of about: 20 (ileum control) and 150 (ileum treated); 60 (glands control) and 300 (glands treated); 200 (heart). Protein concentration was determined by the method of Bradford (39) with dye reagent (Bio-Rad, Richmond, CA). Bovine serum albumin (Fluka, Buchs, Switzerland) was used as standard.

Binding Assays

Control and treated homogenates (1 ml/tube) were incubated for 45 min at 30° with the marker ligand [^{3}H]NMS (equilibrium conditions, as determined by appropriate experiments). Incubation was stopped by rapid filtration under reduced pressure through GF/B glass fiber filters (Whatman, Maidstone, England). Filters were washed with 3 × 5 ml of ice-cold Tris·HCl buffer (pH 7.5) and then introduced into glass scintillation vials. A total of 4 ml of Filter-count (Packard, Downers Grove, IL) was added and radioactivity was determined by liquid scintillation counting.

Specific binding was defined as that displaceable by 1 μM QNB and averaged about 95 and 60% of total binding for control and treated tissues, respectively.

Saturation curves. The homogenates were incubated as indicated above in the presence of various concentrations (0.01–30 nM) of [^{3}H]NMS.

Competition curves. The homogenates were incubated as above in

[1] E. Giraldo and E. Monferini, unpublished observation.

the presence of 0.6 nM [^{3}H]NMS and different concentrations of unlabeled ligands.

Dissociation kinetics. Dissociation of [^{3}H]NMS was started after incubating 0.6 nM [^{3}H]NMS with membrane preparations at 30° for 45 min. At this time (time 0), 1 μM unlabeled NMS was added and determinations were made at various intervals over a 90-min period. In parallel, the stability of the [^{3}H]NMS-receptor complex equilibrium was checked by adding 1 μM unlabeled NMS and filtering immediately thereafter, every 20 min throughout the experiment. The extent of dissociation at various times was calculated as a percentage of [^{3}H] NMS specifically bound (dpm), after 45 min equilibrium (time 0), taking into account the stability of the [^{3}H]NMS-receptor complex equilibrium.

Data Analysis

Saturation and competition curves were analyzed by a nonlinear least squares regression analysis on the basis of a one or a two binding site model with a TOPFIT pharmacokinetic program package (40). Dissociation constants (K_D) for the different unlabeled ligands were obtained after correction for the radioligand occupancy shift, according to the equation $K_D = IC_{50}/1 + [^*C]/^*K_D$ where IC_{50} is the concentration of unlabeled ligand displacing 50% of specific binding and [*C] and $^*K_D$ represent the concentration and the dissociation constant of the radioligand used, respectively (41). K_D values for [^{3}H]NMS in the various tissues under different treatments, as used in the calculations, are found in the tables in Results.

Hill coefficients (n_H) were calculated by linear regression analysis and assessed for significant deviation from unity (Student's t test).

Materials

[^{3}H]NMS (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA); AF-DX 116, PZ, and QNB were synthesized at Dr. K. Thomae GmbH (Biberach an der Riss, FRG); HHSiD was a generous gift from Drs. E. Mutschler and G. Lambrecht (University of Frankfurt, Frankfurt, FRG) and Dr. R. Tacke (University of Braunschweig, Braunschweig, FRG); ATR and NMS were purchased from Sigma (St. Louis, MO) and PBZ was a gift from Smith Kline & French (Philadelphia, PA). All compounds, prepared fresh before use, were dissolved in distilled water.

Results

Cardiac and Glandular Tissues

Displacement experiments. Table 1 gives the binding estimates from curves generated in heart and glands by various muscarinic antagonists (NMS, ATR, PZ, AF-DX 116, and HHSiD) in competition experiments against 0.6 nM [^{3}H]NMS. In each case, the data were well explained by interaction with a single site ($n_H \simeq 1$), indicating that the antagonists bound to a homogeneous population of receptors in both heart and glands. Whereas NMS, ATR, and PZ did not distinguish between the mAChRs present in the two tissues, AF-DX 116 and HHSiD showed a discriminating profile. AF-DX 116 displayed high affinity for the receptors in the heart, with a K_D of 112 ± 13 nM and low affinity for the receptors in the glands, with a K_D of 3982 ± 659 nM. HHSiD demonstrated inverse selectivity, showing high affinity for the glandular receptors, with a K_D of 63 ± 4 nM and low affinity for the cardiac subtype, with a K_D of 499 ± 11 nM.

The binding curves of AF-DX 116 and HHSiD, derived from the competition experiments against 0.6 nM [^{3}H]NMS in membranes from the guinea pig heart and glands, are illustrated in Fig. 2.

Dissociation experiments. Dissociation experiments were performed on these two tissues to investigate the kinetic characteristics of the two receptor subtypes (Fig. 3). After the addition of excess unlabeled NMS (1 μM), a clear difference in the dissociation of [^{3}H]NMS from receptors in the heart with respect to those in the glands was noticed. In cardiac membranes, the dissociation reached 50% in less than 1 min and was completed after 10–20 min; by contrast, in glandular membranes the half-time dissociation was about 20 min, and even after 90 min a detectable amount of [^{3}H]NMS-receptor complex was present.

Smooth Muscle Tissue

Competition experiments. The presence of a heterogeneous population of receptors in guinea pig ileum longitudinal smooth muscle membranes was revealed by displacements carried out against 0.6 nM [^{3}H]NMS with AF-DX 116 and HHSiD (Fig. 4). The AF-DX 116/[^{3}H]NMS competition experiments generated a shallow curve with n_H significantly less than unity. Computer analysis revealed that the data points fitted best to a two binding site model, thus indicating the presence of a mixed population of receptors. AF-DX 116 recognized 65% of total receptors with high affinity (K_D = 64 ± 16 nM) and 35% of receptors with low affinity (K_D = 2097 ± 490 nM) (Table 2).

In HHSiD/[^{3}H]NMS competition experiments, a shallow curve was also generated with an n_H significantly less than unity. According to a two binding site model, HHSiD distinguished 24% of total receptors with high affinity (K_D = 25 ± 5 nM) and 76% of receptors with low affinity (K_D = 355 ± 18 nM) (Table 2).

In Table 2, the values for smooth muscle are compared with affinity values for the compounds in heart and glands. The binding constants of the smooth muscle receptors showing high and low affinity for AF-DX 116 were similar to the respective ones in heart and glands. In the same way, the binding constants of HHSiD for the high and low affinity sites in smooth

TABLE 1

Binding estimates of different mAChR antagonists in heart and submandibular salivary glands of guinea pig

Dissociation constants (K_D) and Hill coefficients (n_H) were determined as described in Materials and Methods. Each value is the mean ± standard error of three to five experiments performed in triplicate.

	Heart		Glands	
	K_D	n_H	K_D	n_H
	nM		nM	
NMS	0.98 ± 0.14	1.05 ± 0.04	0.81 ± 0.08	1.10 ± 0.10
ATR	5.57 ± 0.46	1.06 ± 0.07	6.01 ± 0.29	1.03 ± 0.04
PZ	1246 ± 115	1.03 ± 0.01	1570 ± 178	1.00 ± 0.03
AF-DX 116	112 ± 13*	1.03 ± 0.07	3982 ± 659	0.99 ± 0.06
HHSiD	499 ± 11*	1.01 ± 0.05	63 ± 4	1.03 ± 0.06

* $p < 0.05$ versus glands K_D (Student's t test).

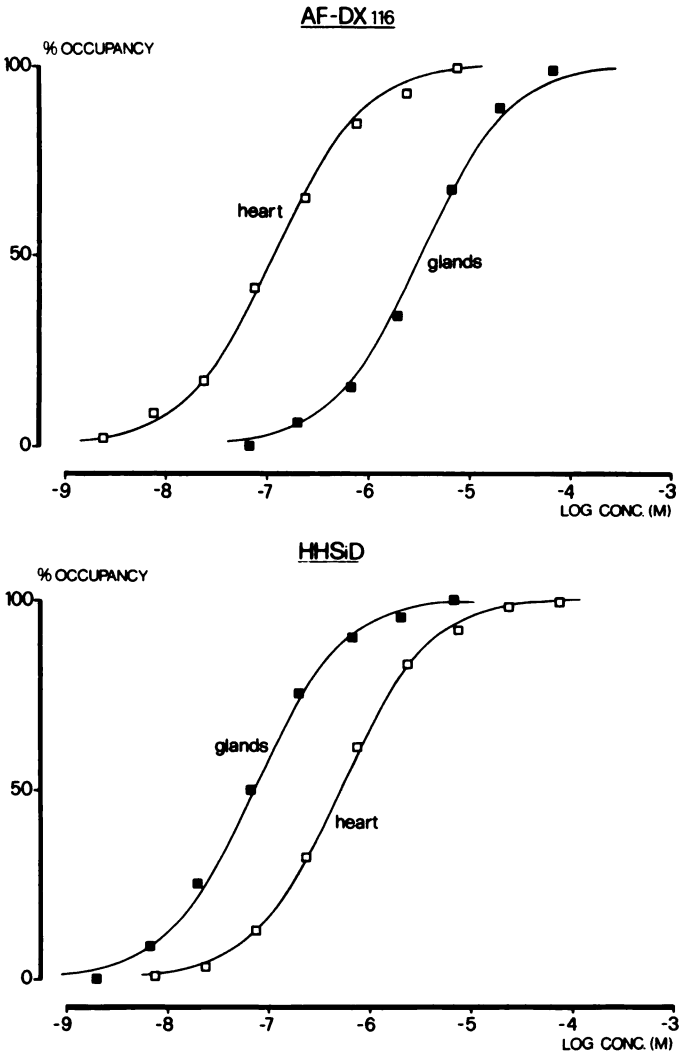

Fig. 2. Binding curves of AF-DX 116 and HHSiD derived from competition experiments against 0.6 nM [^{3}H]NMS in guinea pig cardiac and glandular membranes. Data illustrate a representative experiment performed in triplicate, after correction for the radioligand-induced shift.

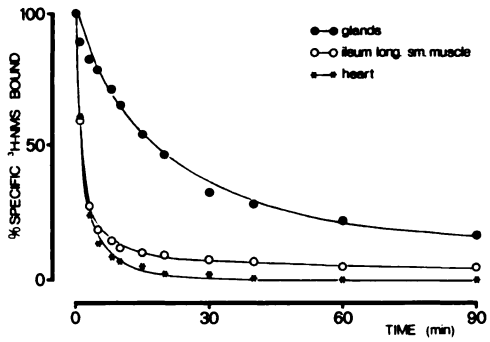

Fig. 3. Time course of 0.6 nM [^{3}H]NMS dissociation from muscarinic receptors of cardiac, ileal, and glandular membranes. The dissociation of [^{3}H]NMS was measured at 30° after incubation with the radioligand to equilibrium for 45 min. At time 0, thereafter, 1 μM cold NMS was added and determinations were made at various times over a 90-min period. Nonspecific binding was defined with 1 μM QNB for each time. Data represent the means of at least three experiments performed in triplicate. Standard errors are within the diameter of the symbols.

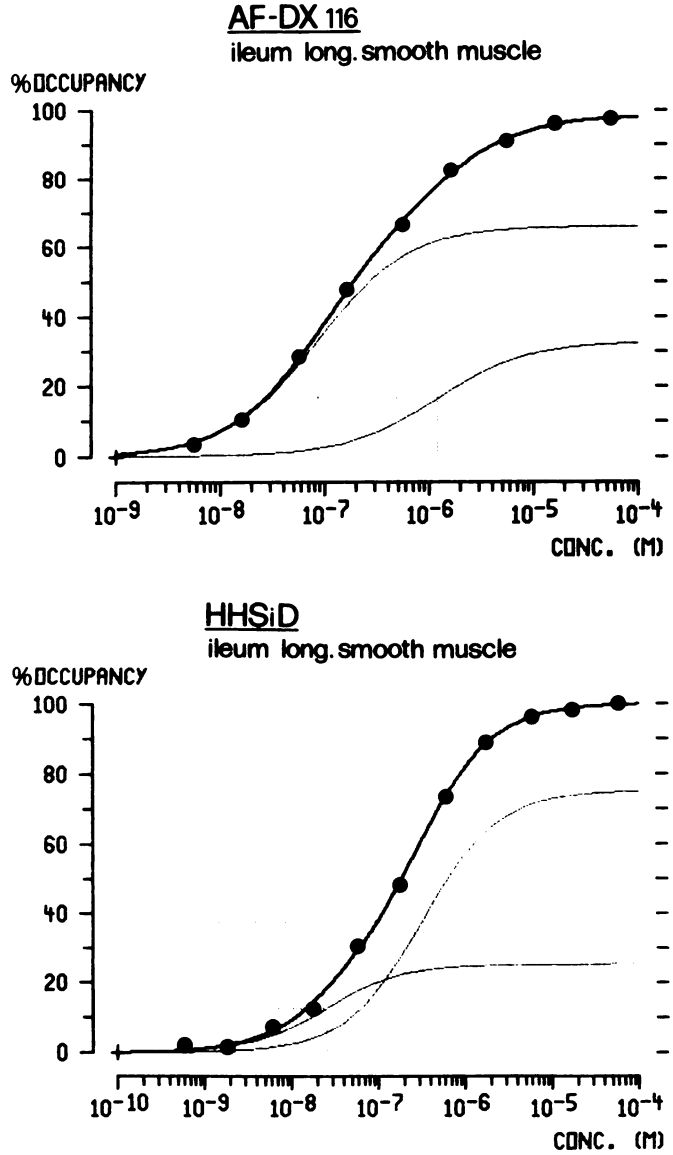

Fig. 4. Binding profile of AF-DX 116 and HHSiD in ileum longitudinal smooth muscle in 0.6 nM [^{3}H]NMS competition experiments. The *curves* indicate the computer fits of the experimental data points of representative experiments after correction for the radioligand occupancy shift, according to a two binding site model with visualization of the individual components of the curves and their dissociation constants.

muscle were quite comparable to those found in glands and heart, respectively. In addition, similar proportions of receptor populations were detected by both antagonists, about 70% cardiac type and 30% glandular type.

Dissociation experiments. In dissociation experiments, the breakdown of the [^{3}H]NMS-receptor complex from the smooth muscle membranes (Fig. 3) was very similar, but not identical, to that for the heart. The first phase was rapid in both tissues, but the amplitude of the first phase was lower and the rate of the second phase was slower for the smooth muscle.

Protection experiments. Saturation studies were carried out in control and treated preparations of guinea pig ileum longitudinal smooth muscle using increasing concentrations of [^{3}H]NMS. The equilibrium constants (K_D) and the total receptor number (R_T) are given in Table 3. Control smooth muscle contained 3225 ± 441 fmol of mAChR/mg of protein with a K_D

TABLE 2

AF-DX 116 and HHSiD binding profile in ileum longitudinal smooth muscle, heart, and submandibular salivary glands of guinea pig

Dissociation constants (K_D, K_{D_1}, and K_{D_2}) were determined as described in Materials and Methods. Hill coefficients are indicated in Table 5 (for ileum longitudinal smooth muscle) and Table 1 (for heart and submandibular salivary glands). Each value is the mean ± standard error of five to six experiments performed in triplicate.

	Ileum longitudinal smooth muscle				Heart K_D	Glands K_D
	K_{D_1}	R_{T_1}	K_{D_2}	R_{T_2}		
	nM	%	nM	%	nM	
AF-DX 116	64 ± 16	65 ± 3	2097 ± 490	35 ± 2	112 ± 13	3982 ± 659
HHSiD	355 ± 18	76 ± 4	25 ± 5	24 ± 4	499 ± 11	63 ± 4

TABLE 3

Equilibrium constant (K_D) and total receptor number (R_T) in control and treated ileum longitudinal smooth muscle of guinea pig, derived from [^{3}H]NMS saturation experiments

The data are the mean ± standard error of at least three experiments each run in triplicate.

	K_D	R_T
	nM	fmol/mg of protein
Control	0.73 ± 0.12	3225 ± 441
Treated (HHSiD/PBZ)	2.17 ± 0.58*	169 ± 18*

*$p < 0.05$ versus respective controls (Student's t test).

TABLE 4

Binding estimates of NMS, ATR, and PZ in control and treated ileum longitudinal smooth muscle of guinea pig

Dissociation constants and Hill coefficients were determined as described in Materials and Methods. Each value is the mean ± standard error of three to five experiments performed in triplicate.

	Control		HHSiD/PBZ	
	K_D	n_H	K_D	n_H
	nM		nM	
NMS	0.92 ± 0.17	0.93 ± 0.04	3.10 ± 0.49*	0.96 ± 0.06
ATR	5.47 ± 0.18	0.99 ± 0.04	19 ± 4.8*	0.99 ± 0.02
PZ	1356 ± 238	0.91 ± 0.03	9289 ± 1281*	0.96 ± 0.13

*$p < 0.05$ versus respective controls (Student's t test).

for [^{3}H]NMS of 0.73 ± 0.12 nM, as determined by nonlinear least squares regression analysis. After protection with HHSiD and treatment with PBZ, the total receptor number was reduced by about 95% to 169 ± 18 fmol of mAChR/mg of protein, and the affinity value for [^{3}H]NMS was significantly increased to 2.17 ± 0.58 nM. This affinity change was also found in displacements with NMS against 0.6 nM [^{3}H]NMS, in which the K_D was increased from 0.92 ± 0.17 nM (control) to 3.10 ± 0.49 nM (treated) ($p < 0.05$) (Table 4). Other studies showed that a rightward shift of the same magnitude in the affinity binding constant for the protected receptor was evident for ATR (from 5.47 ± 0.18 to 19 ± 4.8 nM) and PZ (from 1356 ± 238 to 9289 ± 1281 nM) in displacements against 0.6 nM [^{3}H]NMS (Table 4).

The binding curves for NMS, ATR, and PZ in both control and HHSiD/PBZ-protected tissues were compatible with an interaction at one binding site ($n_H \simeq 1$) (Table 4). On the other hand, whereas AF-DX 116 and HHSiD displaced [^{3}H]NMS with a heterogeneous behavior in the control preparations (n_H values significantly less than unity) (see Fig. 4 and Table 2), in the protected tissue, the experimental data for both compounds followed simple mass action law (n_H values $\simeq 1$) and showed a uniform receptor population with affinity values of 16330 ± 4500 and 217 ± 46 nM for AF-DX 116 and HHSiD, respectively (Table 5).

The difference in binding characteristics between the control and the HHSiD/PBZ-protected preparation was further confirmed in kinetic experiments, in which the off rate of 0.6 nM [^{3}H]NMS from the mAChR in the smooth muscle membrane suspension was evaluated. As illustrated in Fig. 5, [^{3}H]NMS dissociated much more slowly (half-time of dissociation of 30 min) from HHSiD/PBZ-treated receptors than from the control ones, for which the half-time of dissociation was about 1 min. The [^{3}H]NMS off rate behavior from the HHSiD/PBZ-protected smooth muscle receptors was comparable to that from the receptors in the glandular suspension shown in Fig. 3, suggesting in this case too a similarity between the subtype in smooth muscle showing high affinity for HHSiD and the glandular M_3 receptor type.

When the smooth muscle receptors were protected with 10 μM AF-DX 116 instead of 3 μM HHSiD and then treated with PBZ to unmask the cardiac subtype, the dissociation rate for [^{3}H]NMS was somewhat faster than for the control (Fig. 6), revealing kinetic characteristics typical of the cardiac receptor.

Glandular Tissue

Binding curves generated by the classical and selective antagonists in competition experiments against 0.6 nM [^{3}H]NMS in both control and HHSiD/PBZ-protected submandibular salivary glands are well explained by interaction with a single site ($n_H \simeq 1$). Similarly to that found in smooth muscle, the affinity values for all the compounds were shifted to the right by 5- to 10-fold in the protected tissue (Table 6).

Cardiac/Glandular Tissue Mixture

An artificial mixture of 70% cardiac and 30% glandular membranes was treated with HHSiD/PBZ and then used for kinetic studies. As seen in Fig. 7, while the off rate of 0.6 nM [^{3}H]NMS from receptors in the mixed membrane suspension of untreated control reached 50% in less than 2 min, the half-time of dissociation was markedly increased to about 30 min in the treated suspension, suggesting that the receptors in the glandular membranes, but not in the cardiac ones, were protected by HHSiD.

Discussion

Competition binding experiments with the cardioselective antimuscarinic compound AF-DX 116 against [^{3}H]NMS generated a shallow curve in guinea pig ileum longitudinal smooth muscle, indicating the presence of a heterogeneous population of receptors; 65% of the total receptors showed high affinity for AF-DX 116, with a K_D of 64 nM, and the remainder, 35%, bound the cardioselective antagonist with low affinity, displaying a K_D of 2097 nM. In guinea pig heart and submandibular salivary glands, AF-DX 116 recognized a uniform population

TABLE 5

AF-DX 116 and HHSiD binding profile in control and treated ileum longitudinal smooth muscle of guinea pig

Dissociation constants and Hill coefficients were determined as described in Materials and Methods. Each value is the mean ± standard error of five to six experiments performed in triplicate.

	Control			HHSiD/PBZ	
	K_{D1}	K_{D2}	n_H	K_D	n_H
	nM			nM	
AF-DX 116	64 ± 16	2097 ± 490	0.74 ± 0.03*	16330 ± 4500	0.99 ± 0.04
HHSiD	355 ± 18	25 ± 5	0.84 ± 0.03*	217 ± 46	0.90 ± 0.04

* n_H significantly less than 1 ($p < 0.05$) (Student's t test).

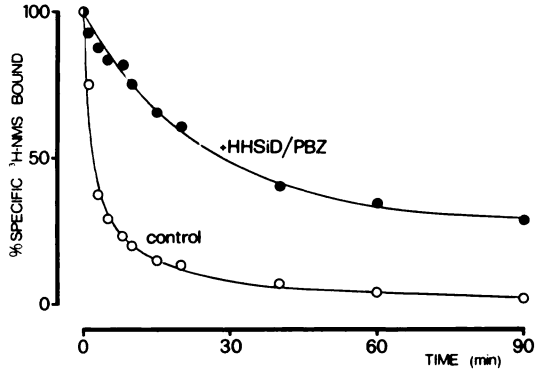

Fig. 5. [³H]NMS dissociation from ileum longitudinal smooth muscle membranes as control and after HHSiD/PBZ treatment. After an incubation of 45 min at 30° with 0.6 nM [³H]NMS, 1 μM cold NMS was added and determinations were made at various times over a period of 90 min. Nonspecific binding was defined with 1 μM QNB at each time point. Data represent the means of at least three experiments performed in triplicate. Standard errors are within the symbols.

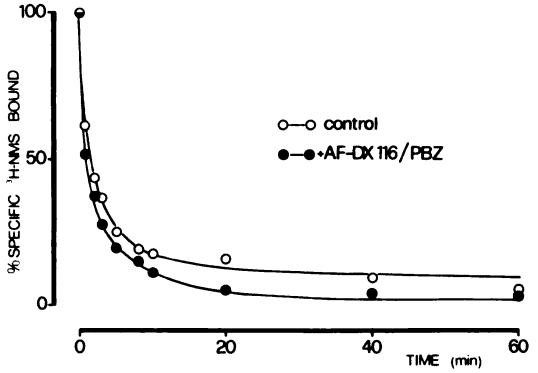

Fig. 6. [³H]NMS dissociation from ileum longitudinal smooth muscle muscarinic receptors of untreated control and AF-DX 116/PBZ-treated membranes. Details are given in the legend to Fig. 5.

of sites exhibiting a selective profile; it bound with high affinity (K_D of 112 nM) to cardiac membranes and with low affinity (K_D of 3982 nM) to glandular membranes. The K_D values for the two subtypes found in longitudinal smooth muscle, when compared with the respective K_D values in heart and glands, were close enough to suggest that these subtypes may be considered cardiac M_2 and glandular M_3 receptors, as already described (32, 33).

The evidence for the presence of two different receptor populations in smooth muscle was supported by data obtained using HHSiD, a selective compound demonstrated to exhibit a 30-fold selectivity for smooth muscle versus atrium in isolated organ preparations (35, 36). The K_D values obtained from [³H]NMS/HHSiD competition experiments in smooth muscle (25 and 355 nM), again, were comparable to the respective K_D values found in glands (63 nM) and in heart (449 nM). It is clear from the data that AF-DX 116 and HHSiD possess inverse binding characteristics, the former being cardioselective, the latter glandular selective. Both drugs revealed that approximately 70% of receptors in smooth muscle showed similarity to the cardiac M_2 and 30% emulated the glandular M_3 types.

Additional evidence attesting to this phenomenon was furnished by the kinetic experiments, valuable tools for bringing out differences between receptors (42, 43). The rate of breakdown of the [³H]NMS-receptor complex in smooth muscle membranes was close to, but not overlapping with, that of the heart. This finding may be due to the existence of a new muscarinic receptor subtype (M_4?), because the presence of a significant amount of the M_1 subtype has to excluded from binding and functional data (26, 29). Alternatively, the differences in the dissociation curves may be due to the described heterogeneity (cardiac plus glandular subtypes) of the smooth muscle receptors, and the higher proportion of the cardiac subtype may account for the closeness of the dissociation curve to that of the heart.

The presence of heterogeneous populations of receptors in the smooth muscle preparation does not alone show that they

TABLE 6

Binding estimates of different mAChR antagonists in control and treated submandibular salivary glands of guinea pig

Dissociation constants and Hill coefficients were determined as described in Materials and Methods. Each value is the mean ± standard error of at least three experiments performed in triplicate.

	Control		HHSiD/PBZ	
	K_D	n_H	K_D	n_H
	nM		nM	
NMS	0.81 ± 0.08	1.10 ± 0.10	4.02 ± 0.33*	0.97 ± 0.08
ATR	6.01 ± 0.29	1.03 ± 0.04	32 ± 4.10*	1.02 ± 0.09
PZ	1570 ± 178	1.00 ± 0.03	13653 ± 342*	1.08 ± 0.04
AF-DX 116	3982 ± 659	0.99 ± 0.06	31940 ± 5500*	0.89 ± 0.07
HHSiD	63 ± 4.07	1.03 ± 0.06	647 ± 87*	1.06 ± 0.07

* $p < 0.05$ versus respective controls (Student's t test).

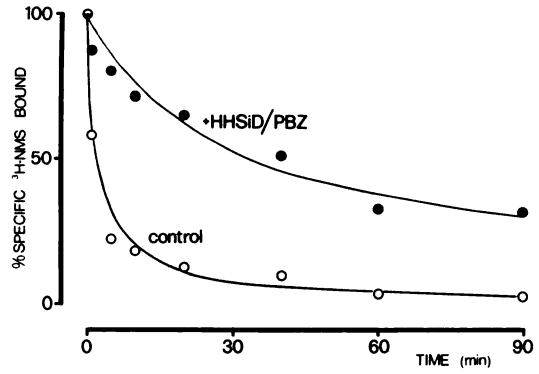

Fig. 7. Dissociation of 0.6 nM [^{3}H]NMS from muscarinic receptors of untreated control artificial mixture of 30% glandular and 70% cardiac membranes and after treatment with HHSiD/PBX. Details are given in the legend to Fig. 5.

are intrinsic to the muscle cells. It cannot be readily excluded that some receptors are localized to membranes of other cell types (e.g., neuronal cells from Auerbach's plexus or endothelial cells), which are likely present in the preparation. On the other hand, we have evidence that the total mAChR number in synaptosomes from the myenteric plexus amounted to 23.5 fmol/mg of protein (20) or less than 1% of the total mAChR number (3225 fmol/mg of protein) in the smooth muscle membranes. Although receptor presence on endothelial cells cannot be excluded, some preliminary binding studies[1] performed with AF-DX 116 on isolated smooth muscle cells in competition against [^{3}H]NMS have confirmed heterogeneity, indicating localization of the receptor subtypes to the muscle. Whereas Roffel *et al.* (17) concluded that the glandular subtype they found in tracheal smooth muscle was localized in the submucosal glands, this is not the case in our longitudinal muscle preparation from which the mucosa was removed.

The property of HHSiD to distinguish the functional mAChRs in smooth muscle from those in atria in *in vitro* isolated organ preparations (pA_2 of 7.96 versus 6.53–6.83, respectively) (36) and to discriminate between glandular M_3 and cardiac M_2 types in binding (K_D of 63 versus 499 nM, respectively) was exploited to selectively protect the higher affinity sites present in the longitudinal smooth muscle. The protection of these sites was achieved by preincubation with an appropriate concentration of HHSiD followed by treatment with the alkylating agent PBZ to irreversibly block the remaining unprotected sites. Saturation experiments indicated that effectively less than 10% of the total amount of receptors was available after this treatment. To verify the nature of the protected receptors, displacement studies with different classical and selective antagonists were performed. The first result to emerge from these experiments was the change of the shape of the displacement curves generated by AF-DX 116 and HHSiD. In fact, they were modified from shallow in control membranes to steep in protected ones and, in this case, they could be sufficiently described by a one binding site model. This suggested a uniformity in the receptor population investigated. Although this finding was to be expected, because the purpose of the experiment was to eliminate the low affinity sites for HHSiD and work with only the high affinity glandular subtype of receptors, an unexpected result was the rightward shift of the affinity values for AF-DX 116 and HHSiD in the treated membranes. Starting from the assumption that the protected receptor had glandular characteristics, we would have expected to find a low affinity site for AF-DX 116 with a K_D of approximately 2000–4000 nM and a high affinity site for HHSiD with a K_D of approximately 30–60 nM rather than the K_D values found, which were 4- to 8-fold higher for each compound. This rightward shift of about the same extent was observed, too, for other antagonists, i.e., NMS, ATR, and PZ.

To find an explanation for this phenomenon, displacement experiments with the ligands were repeated in glandular membranes treated with HHSiD/PBZ. Because a similar rightward shift in the curves generated by all the tested antagonists was found in this tissue, too, which contains a uniform population of mAChRs, it appears that the artificial creation of a new type of receptor in smooth muscle by the HHSiD/PBZ treatment could be ruled out. Other proof substantiating that the HHSiD/PBZ treatment protected a receptor population already existent in this tissue was furnished by the kinetic experiments. In this condition, the protected receptor exhibited an off rate profile quite similar to that of the glandular type. This evidence confirmed that the HHSiD/PBZ treatment was able, indeed, to protect a glandular receptor population present in this tissue.

In order to exclude a possible influence of the experimental procedure on the kinetic properties of the receptor(s), we decided to characterize in the same way the cardiac receptor subtype present in the smooth muscle by using an AF-DX 116/PBZ treatment. As expected, we found that this differently protected receptor possessed the same kinetic characteristics as the pure cardiac subtype, with [^{3}H]NMS dissociating faster from the treated than from the control membranes. It is thus demonstrated that the receptor protection with selective compounds, followed by an irreversible alkylating treatment, is a suitable procedure to obtain a homogeneous receptor population, validating our results with the smooth muscle.

The hypothesis of the presence of 70% cardiac type and 30% glandular type in smooth muscle was further confirmed by other kinetic studies performed with an artificial mixture of the two pure receptor populations. In fact, a combination of 70% cardiac and 30% glandular membranes, subjected to the same HHSiD/PBZ procedure, showed a dissociation profile quite superimposable on that found in smooth muscle. This gives the first direct indication of the presence of both cardiac and glandular receptor subtypes in the smooth muscle membranes. Displacement studies with this mixture may be helpful in confirming this assessment.

It has been reported (44) that methoctramine, a tetraamine derivative described to have higher affinity for cardiac muscarinic receptors both in functional and binding studies (45, 46), recognized two receptor populations in rat longitudinal and circular smooth muscles in competition binding experiments against [^{3}H]NMS, a cardiac type (70–80% of total receptors) and a glandular type (20–30% of total receptors). The authors showed in the same way that AF-DX 116 exhibited a heterogeneous binding profile in circular but not in longitudinal smooth muscle. This discrepancy with our results can likely be explained on the basis of different experimental conditions because we were able to find the concomitance of cardiac and glandular receptor subtypes in various intestinal smooth muscles of guinea pig, rat, and rabbit (33) and in urinary bladder of rat (34) with AF-DX 116.

A significant consequence of these results is to verify which of the two receptor populations, or which combination of the two, is responsible for the muscarinic agonist-stimulated

smooth muscle contraction. From a direct correlation between *in vitro* binding data and *in vitro* functional results with the smooth muscle-selective compound, HHSiD, and the cardioselective one, AF-DX 116, we are led to believe that the receptor mediating the contraction belongs to the glandular population. In fact, HHSiD and AF-DX 116, which in functional tests (11, 16, 35, 36) have, respectively, high and low potency in blocking muscarinic agonist-mediated ileal contraction, show in binding studies a comparable affinity for the glandular receptor. The hypothesis is further supported by studies performed with other compounds such as 4-DAMP, CPPS, and methoctramine. 4-DAMP and CPPS, ileum-selective compounds in functional studies (28, 30, 47), exhibited high binding affinity for the glandular receptor (34, 44, 48). Methoctramine, a cardioselective compound with low potency in inhibiting ileal contraction (45), has low affinity for the glandular receptor (44, 46).

The role that the cardiac subtype plays in smooth muscle cell function is unclear. Answers as to whether it is accessible to agonists and whether it is externalized during the homogenization process, while normally it is down-regulated by endogeneous ACh, will shed some light on its function in contractility mechanisms. However, it is interesting to note that Burgen and Spero (49), studying the effect of various agonists and antagonists on two different functional responses (contraction and Rb^+ efflux), suggested that there are present in guinea pig ileum two types of muscarinic receptors, "one possibly located in the vicinity of the neural endings and the other elsewhere, and that these receptors are distinguishable in their responsiveness to muscarinic agonists" (p. 114).

Acknowledgments

We gratefully acknowledge Ms. Luciana Monti for technical assistance, Ms. Carla Pelanda for statistical assistance, and Ms. Luciana Spagnuolo for the excellent typing of the manuscript. We thank Dr. Loris De Conti for the helpful discussions.

References

1. Engel, W., W. Eberlein, G. Trumlitz, and G. Mihm. Pirenzepine and AF-DX 116, two structurally related antimuscarinics with opposite receptor selectivity. *Fed. Proc.* **46**:2527–2528 (1987).
2. Goyal, R. K., and S. Rattan. Neurochemical, hormonal and drug receptors for the lower oesophageal sphincter. *Gastroenterology* **74**:598–619 (1978).
3. Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature (Lond.)* **283**:90–92 (1980).
4. Hammer, R., and A. Giachetti. Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterization. *Life Sci.* **31**:2991–2998 (1982).
5. Hirschowitz, B. I., R. Hammer, A. Giachetti, J. J. Keirns, and R. R. Levine, eds. *Trends in Pharmacological Sciences.* Elsevier, Amsterdam. 1984 Vol. 5 (suppl.): *Subtypes of Muscarinic Receptors.*
6. Levine, R. R., N. J. M. Birdsall, A. Giachetti, R. Hammer, L. L. Iversen, D. J. Jenden, and R. A. North, eds. *Trends in Pharmacological Sciences.* Elsevier, Amsterdam. 1986 Vol. 7 (suppl.): *Subtypes of Muscarinic Receptors II.*
7. Levine, R. R., N. J. M. Birdsall, R. A. North, M. Holman, A. Watanabe, and L. L. Iversen, eds. *Trends in Pharmacological Sciences.* Elsevier, Amsterdam. 1988 Vol. 9 (suppl.): *Subtypes of Muscarinic Receptors III.*
8. Nathanson, N. M. Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.* **10**:195–236 (1987).
9. Giachetti A., E. Giraldo, H. Ladinsky, and E. Montagna. Binding and functional profiles of the selective M_1 muscarinic receptor antagonists trihexyphenidyl and dicyclomine. *Br. J. Pharmacol* **89**:83–90 (1986).
10. Hammer, R., E. Giraldo, G. B. Schiavi, E. Monferini, and H. Ladinsky. Binding profile of a novel cardioselective muscarine receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci.* **38**:1653–1662 (1986).
11. Giachetti, A., R. Micheletti, and E. Montagna. Cardioselective profile of AF-DX 116, a muscarinic M_2 receptor antagonist. *Life Sci* **38**:1663–1672 (1986).
12. El-Fakahany, E. E., V. Ramkumar, and W. S. Lai. Multiple binding affinities of *N*-methylscopolamine to brain muscarinic acetylcholine receptors: differentiation from M_1 and M_2 receptors subtypes. *J. Pharmacol. Exp. Ther.* **238**:554–563 (1986).
13. Watson, M., W. R. Roeske, and H. I. Yamamura. ^{3}H-Pirenzepine and ^{3}H-quinuclidinyl benzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. II. Characterization and regulation of antagonist binding to putative muscarinic subtypes. *J. Pharmacol. Exp. Ther.* **237**:419–427 (1986).
14. Korc, M., M. S. Ackerman, and W. R. Roeske. A cholinergic antagonist identifies a subclass of muscarinic receptors in isolated rat pancreatic acini. *J. Pharmacol. Exp. Ther.* **240**:118–122 (1987).
15. Giraldo, E., R. Hammer, and H. Ladinsky. Distribution of muscarinic receptor subtypes in rat brain as determined in binding studies with AF-DX 116 and pirenzepine. *Life Sci.* **40**:833–840 (1987).
16. Micheletti, R., E. Montagna, and A. Giachetti. AF-DX 116, a cardioselective muscarinic antagonist. *J. Pharmacol. Exp. Ther.* **241**:628–634 (1987).
17. Roffel, A. F., W. G. in't Hout, R. A. de Zeeuw, and J. Zaagsma. The M_2 selective antagonist AF-DX 116 shows high affinity for muscarinic receptors in bovine tracheal membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**:593–595 (1987).
18. Martos, F., E. Monferini, E. Giraldo, A. M. De Paoli, and R. Hammer. Characterization of muscarinic receptors in salivary and lacrimal glands of the rat. *Eur. J. Pharmacol.* **143**:189–194 (1987).
19. Giraldo, E., E. Monferini, and R. Hammer. Selective labelling of M_1 receptors in autonomic ganglia with ^{3}H-pirenzepine. *Arzneim.-Forsch. Drug. Res.* **35**:325–328 (1985).
20. Giachetti, A., E. Monferini, A. Schiavone, R. Micheletti, R. Hammer, and H. Ladinsky. Functional and biochemical evidence for muscarinic receptor subtypes in the gastrointestinal tract, in *Muscarinic Receptor Subtypes in the GI Tract* (G. Lux and E. E. Daniel, eds.). Springer-Verlag, Berlin, 14–19 (1985).
21. Doods, H. N., M. J. Mathy, D. Davidesko, K. J. van Charldorp, A. de Jonge, and P. A. van Zwieten. 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**:257–262 (1987).
22. Ladinsky, H., E. Giraldo, G. B. Schiavi, E. Monferini, and R. Hammer. Biochemical characteristics of the cardioselective muscarinic antagonist AF-DX 116. *Fed. Proc.* **46**:2528–2529 (1987).
23. Kubo, T., K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature (Lond.)* **323**:411–416 (1986).
24. Peralta, E. G., J. W. Winslow, J. L. Peterson, D. H. Smith, A. Ashkenazi, J. Ramachandran, M. I. Schimerlik, and D. J. Capon. Primary structure and biochemical properties of an M_2 muscarinic receptor. *Science (Wash. DC)* **236**:600–605 (1987).
25. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetylcholine receptor genes. *Science (Wash. DC)* **237**:527–532 (1987).
26. Hammer, R. Subclasses of muscarinic receptors and pirenzepine: further experimental evidence. *Scand. J. Gastroenterol.* **17**(suppl. 72):59–67 (1982).
27. Halim, S., H. Kilbinger, and I. Wessler. Pirenzepine does not discriminate between pre- and post-synaptic muscarinic receptors in the guinea pig small intestine. *Scand. J. Gastroenterol.* **17**(suppl. 72):87–94 (1982).
28. Clague, R. V., R. M. Eglen, A. C. Strachan, and R. L. Whiting. Action of agonists and antagonists at muscarinic receptors present on ileum and atria in vitro. *Br. J. Pharmacol.* **86**:163–170 (1985).
29. Konno, F., and I. Takayanagi. Comparison of the muscarinic cholinoceptors in the rabbit ciliary body and the guinea pig ileum. *Eur. J. Pharmacol.* **132**:171–178 (1986).
30. Barlow, R. B., K. Y. Berry, P. A. M. Glenton, N. M. Nikolau, and K. S. Soh. A comparison of affinity constants for muscarinic sensitive acetylcholine receptors in guinea pig atrial pace-maker cells at 29°C and in ileum at 29°C and 37°C. *Br. J. Pharmacol.* **58**:613–620 (1976).
31. Eglen, R. M., B. A. Kenny, A. D. Michel, and R. L. Whiting. Muscarinic activity of McN-A-343 and its value in muscarinic receptor classification. *Br. J. Pharmacol.* **90**:693–700 (1987).
32. Giraldo, E., E. Monferini, H. Ladinsky, and R. Hammer. Muscarinic receptor heterogeneity in guinea pig intestinal smooth muscle: binding studies with AF-DX 116. *Eur. J. Pharmacol.* **141**:475–477 (1987).
33. Ladinsky, H., E. Giraldo, E. Monferini, G. B. Schiavi, M. A. Viganò, L. De Conti, R. Micheletti, and R. Hammer. Muscarinic receptor heterogeneity in smooth muscle: binding and functional studies with AF-DX 116. *Trends Pharmacol Sci.* (suppl.) 44–48 (1988).
34. Monferini, E., E. Giraldo, and H. Ladinsky. Characterization of the muscarinic receptor subtypes in the rat urinary bladder. *Eur. J. Pharmacol.*, **147**:453–458 (1988).
35. Mutschler, E., and G. Lambrecht. Selective muscarinic agonists and antagonists in functional tests. *Trends Pharmacol. Sci.* **5**(suppl.):39–44 (1984).
36. Lambrecht, G., and E. Mutschler. Selective inhibition of muscarinic receptors in intestinal smooth muscle, in *Muscarinic Receptor Subtypes in the GI Tract* (G. Lux and E. E. Daniel, eds.). Springer-Verlag, Berlin, 20–27 (1985).
37. Morgenstern, R., and R. Winter. The allosteric influence of β-haloalkylamines on the acetylcholine receptor of smooth muscle. *Acta Biol. Med. Ger.* **34**:K1–K5 (1975).
38. Rang, H. P. Stimulant action of volatile anaesthetics on smooth muscles. *Br. J. Pharmacol.* **22**:356–365 (1964).
39. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).

40. Heinzel, G. Pharmacokinetics during drug development: data analysis and evaluation techniques (G. Bozler and J. M. Van Rossum, eds.) G. Fisher, New York, 207–215 (1982).
41. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
42. Waelbroeck, M., M. Gillard, P. Robberecht, and J. Christophe. Kinetic studies of ^{3}H *N*-methylscopolamine binding to muscarinic receptors in the rat central nervous system: evidence for the existence of three classes of binding sites. *Mol. Pharmacol.* **30**:305–311 (1986).
43. Hammer, R., E. Monferini, L. De Conti, E. Giraldo, G. B. Schiavi, and H. Ladinsky. Subclasses of M_2 muscarinic receptors, in *Cellular and Molecular Basis of Cholinergic Function* (M. J. Dowdall and J. N. Hawthorne, eds.). Verlagsgesellschaft, Weinheim, FRG 56–63 (1987).
44. Michel, A. D., and R. L. Whiting. Methoctramine reveals heterogeneity of M_2 muscarinic receptors in longitudinal ileal smooth muscle membranes. *Eur. J. Pharmacol.* **145**:305–311 (1988).
45. Melchiorre, C., A. Cassinelli, and W. Quaglia. Differential blockade of muscarinic receptor subtypes by polymethylene tetraamines: novel class of selective antagonists of cardiac M_2 muscarinic receptors. *J. Med. Chem.* **30**:201–204 (1987).
46. Giraldo, E., R. Micheletti, E. Montagna, A. Giachetti, M. A. Viganò, H. Ladinsky, and C. Melchiorre. Binding and functional characterization of the cardioselective muscarinic antagonist methoctramine. *J. Pharmacol. Exp. Ther.* **244**:1016–1020 (1988).
47. Eglen, R. M., and R. L. Whiting. Differential affinities of muscarinic antagonists at ileal and atrial receptors. *Br. J. Pharmacol.* **87**(suppl.):33P (1986).
48. Berrie, C. P., N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. The binding of muscarinic receptors in the lacrimal gland: comparison with the cerebral cortex and myocardium. *Br. J. Pharmacol.* **78**:66P (1983).
49. Burgen, A. S. V., and L. Spero. The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth muscle of the guinea-pig intestine. *Br. J. Pharmacol.* **34**:99–115 (1968).

Send reprint requests to: E. Giraldo, Department of Biochemistry, Istituto De Angeli S.p.A., P.O. Box 13059, 20139 Milan, Italy.