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Selectivity of LG50643 for Postjunctional Muscarinic-receptor Subtype in the Guinea-pig Trachea

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Abstract—The effects of (±)-LG50643, a new *N*-quaternary tropinic ester of phenylcyclohexene carboxylic acid, endowed with a potent antimuscarinic activity, have been investigated on muscarinic receptor-mediated responses of the guinea-pig trachea to electrical field stimulation. An isolated preparation which allows the simultaneous measurement of tritiated acetylcholine release (prejunctional effect) and smooth muscle contraction (postjunctional effect) was used. The guinea-pig epithelium-deprived trachea was stimulated with 500 pulses (20 Hz, 1 ms, 9 V for 5 s, 30 s apart) in the presence of indomethacin (1 μM). Three successive pre- and postjunctional responses were observed. The potencies (−logEC50) of (±)-LG50643 for pre- and postjunctional muscarinic receptors were determined and compared with those of selective muscarinic antagonists. In addition, the affinity values of (±)-LG50643 for muscarinic-receptor subtypes were determined in radioligand binding experiments in cerebral cortex, heart and salivary glands of rat as target tissues for M_1, M_2 and M_3 receptors, respectively. The results obtained in both functional and binding assays indicate (±)-LG50643 is a potent and selective antagonist for the M_3-receptor subtype.

Muscarinic receptors can be discriminated by selective antagonists and, accordingly, subdivided into at least three pharmacological subtypes (M_1, M_2 and M_3; for review see Hulme et al (1990)). The selective muscarinic antagonists pirenzepine (Hammer et al 1980), AF-DX116 (Giachetti et al 1986), methoctramine (Giraldo et al 1988), hexahydrosiladiphenidol (HHSiD) and its *p*-fluoro analogue (*p*-F-HHSiD) (Lambrecht et al 1989) have proved to be useful tools in this subclassification in both functional and radioligand binding studies. Recently, a fourth subtype, a putative M_4 receptor, with high affinity for methoctramine and himbacine as well as for HHSiD and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) has been suggested in NG108-15 cells (Michel et al 1989), rat forebrain (Waelbroeck et al 1990) and rabbit lung (Lazareno et al 1990).

It is widely accepted that muscarinic-receptor subtypes have different tissue distribution; in particular, M_1 receptors are located in parasympathetic ganglia and sympathetic nerves, M_2 receptors predominate in heart and on cholinergic nerves (autoreceptors) and M_3 receptors are present in salivary glands and smooth muscle. In addition to this heterogeneity in tissue distribution, there is increasing evidence that a heterogeneous muscarinic receptor population may endow a cholinergically innervated tissue. It has been reported recently that muscarinic receptors localized at the two sides of the cleft in cholinergic nerve terminals of the guinea-pig (Kilbinger et al 1991; Yang & Biggs 1991; Watson et al 1992) and rabbit (Loenders et al 1992), and in rat airways (Aas & Maclagan 1990) belong to different subtypes.

Guinea-pig trachea release experiments demonstrated that the inhibition of acetylcholine release is mediated via a prejunctional muscarinic M_2-like autoreceptor and confirmed that the activation of postjunctional M_3 receptors caused smooth muscle contraction (Kilbinger et al 1991). During cholinergic nerve stimulation non-selective muscarinic antagonists, such as scopolamine or ipratropium bromide, counteract smooth muscle contraction induced by released acetylcholine, but at the same time they increase the overflow of acetylcholine through the inhibition of the negative feedback system. Such an enhanced output may overcome postjunctional blockade, thus making these non-selective antagonists less effective in bronchodilatation than a selective antagonist of M_3 receptors. Because bronchoconstriction may be relevant in airway obstruction (Maclagan & Barnes 1989), new M_3-subtype-preferring antagonists might have therapeutic relevance in the treatment of obstructive airway disease.

(±)-LG50643, (±)-3[[(2-phenyl-2-cycloexene-1-yl)carbonyl]oxy]-8,8-diethyl-8-azoniabicyclo[3.2.1.]octane iodide (Fig. 1), is a new *N*-quaternary tropinic ester of phenylcyclohexene carboxylic acid which appeared to be endowed with a potent antimuscarinic activity in pharmacological models (Subissi & Criscuoli 1987; Turbanti et al 1992). The objective of the present study was to assess the pharmacological profile of (±)-LG50643 for subtypes of muscarinic receptors in the airways.

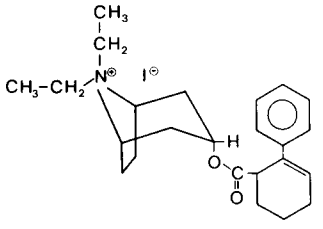

FIG. 1. Structural formula of (±)-LG50643.

For this purpose the pre- and postjunctional potencies of (±)-LG50643 were compared with those of five subtype-preferring muscarinic antagonists on an isolated preparation

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Table 1. Methodological details for binding experiments in rat tissues.

Tissue receptor	Cerebral cortex (M_1)	Heart (M_2)	Salivary glands (M_3)
Incubation buffer	50 mM Na/K phosphate (pH 7·4)	10 mM Tris-HCl (pH 7·5)	20 mM HEPES (pH 7·5)
Additions	10 mM $MgCl_2$	1 mM EDTA	10–100 mM $MgCl_2$
Membrane concn (mg wet tissue/1 mL incubation)	0·5–1	1–2	1–3
[^{3}H]QNB (nM)	0·23	0·4	0·4–0·5
Incubation time (min)	60	60	45
Temperature (°C)	25	37	30

of guinea-pig trachea which allows the simultaneous measurement of acetylcholine release (prejunctional effect) and smooth-muscle contraction (postjunctional effect) (D'Agostino et al 1990; Kilbinger et al 1991). The rank order of the postjunctional potencies of the five antagonists as well as the postjunctional pA_2 values were also compared. In addition, the affinity values of (±)-LG50643, 4-DAMP and *p*-F-HHSiD for muscarinic receptors were calculated in radioligand binding assays. Competition experiments were carried out in cerebral cortex, heart and salivary glands of rat as target tissues for M_1, M_2 and M_3 receptors, respectively.

Materials and Methods

Drugs

[Methyl-^{3}H]choline and [^{3}H]QNB (quinidylbenzilate) were obtained from Amersham (Buckinghamshire, UK). Methoctramine hydrochloride, 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide), *p*-F-HHSiD (*p*-fluorohexahydrosiladiphenidol hydrochloride), telenzepine dihydrochloride were from RBI (Natick, MA, USA). (±)-LG50643 was synthesized at Laboratori Guidotti by Professor L. Turbanti. All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Tissue preparation

Male albino guinea-pigs (Dunkin-Hartley strain, 500–700 g) were killed by a blow to the head and exsanguinated. The trachea was rapidly removed and gently cleaned of adhering connective tissue in a prewarmed (37°C) and oxygenated (95% O_2–5% CO_2) medium of the following composition (mM): NaCl 118, KCl 5·6, $CaCl_2$ 2·5, $MgSO_4$ 1·19, $NaHCO_3$ 25·0, NaH_2PO_4 1·3, glucose 10. The epithelium was removed completely by rubbing the luminal surface with a moistened pipe-cleaner. A clip-connected strip was then prepared from one trachea as described in detail by D'Agostino et al (1990).

Outflow of [^{3}H]acetylcholine

During a 30 min equilibration period, the strip was suspended isometrically in superfusional medium (2 mL min^{-1}) under a tension of 1 g and stimulated electrically with trains of impulses of 1 ms duration delivered at 10 Hz for 5 s, 30 s apart with a voltage drop between the parallel electrodes of 9 V cm^{-1}. Superfusion was then stopped and the preparation incubated for 60 min with [^{3}H]choline (92·5 KBq; final concentration 1 μM) under field stimulation at 20 Hz (parameters as above). At the end of the labelling period the strip was superfused with medium that contained in addition hemicholinium-3 (10 μM) and indomethacin (1 μM). Indomethacin was used to prevent a predominantly inhibitory effect by prostaglandins of both cholinergic neurotransmission and contractile response (Walters et al 1984; Brunn et al 1992). After a 120-min wash-out, the superfusate was collected continuously in 3-min fractions and measured for ^{3}H content by liquid scintillation spectrometry. Three stimulation periods (S_1, S_2 and S_3) were carried out 30 min apart on each preparation, the first starting 9 min after the end of the wash-out period. Each electrical stimulation consisted of 500 pulses applied in trains of 100 pulses (1 ms duration) at a frequency of 20 Hz in intervals of 30 s. The stimulation-evoked outflow of ^{3}H was calculated from the difference between the total outflow during the stimulation plus the following 9 min and the estimated basal outflow. The basal outflow was assumed to decline linearly from the three samples before and the three samples 15–24 min after the onset of the stimulation. The basal and the electrically-evoked outflows are expressed in disintegration s^{-1} (g tissue)$^{-1}$.

Pre- and postjunctional potencies of antagonists

Muscarinic antagonists were added in the perfusional medium in increasing concentrations, 18 min before S_2 and S_3 and their prejunctional effect was expressed as ratios S_n/S_1 (S_n:S_2–S_3).

Contractions of the smooth muscle were recorded simultaneously with a force displacement transducer and displayed on a Battaglia-Rangoni recorder. The peak tension developed by all twitches during S_1 (postjunctional effect) were summed, and this value was compared with that obtained by summing the individual responses in S_2 and S_3.

Complete concentration-response curves for pre- and postjunctional effects were constructed by expressing the ratio S_n/S_1 in the presence of an antagonist as a percentage of the equivalent ratio obtained in the absence of antagonists in control experiments. The negative logarithm of the concentration which produced half-maximal responses (−log EC50) was determined graphically from individual concentration-response curves.

Determination of postjunctional pA_2 values

After the epithelium removal, two zig-zag strips were obtained from each trachea (Emmerson & Mackay 1979). The preparation was suspended isometrically under a tension of 1 g during an equilibrium period of 1 h in a physiological salt solution (composition as above) at 37°C containing 1 μM indomethacin. Cumulative concentration-response curves to carbachol were then carried out in the absence or in the presence of the antagonist and corrected for any changes in sensitivity to carbachol (Grana et al 1986). An exposure of 30 min to the antagonist was allowed. Only one dose of antagonist was used in the same preparation. The pA_2 values were determined from Schild plots by regression analysis (Arunlakshana & Schild 1959).

Radioligand binding assay

Tissues of rats (Charles River strain, 200–300 g) were rapidly dissected from exsanguinated animals, rinsed in cold saline and immediately used or stored at −20°C after freezing in liquid nitrogen. Membranes were prepared according to the procedure described in detail by Renzetti et al (1990). In brief, fresh or thawed tissues were homogenized in suitable buffer by means of an Ultraturrax homogenizer. Crude homogenates were centrifuged and the pellets were resuspended in an appropriate volume of incubation buffer to obtain the tissue concentrations desired for the test. The experimental conditions for binding studies, revealed to be the most suitable within the three different receptor preparations in preliminary trials, are reported in Table 1. The concentrations of muscarinic antagonists ranged from 0·01 nM to 100 μM and non-specific binding was determined in the presence of 1 μM atropine as unlabelled ligand. Non-specific binding usually averaged 10–20% of the total binding. Reactions were determined by filtering the samples under reduced pressure through glass fibre filters (Whatman GF/B). The filters were then soaked in 0·01 M KOH. Overnight equilibration at room temperature (21°C) was followed by radioactivity counting in a scintillation counter.

Statistical analysis of results

The K_D (equilibrium dissociation constant), B_{max} (maximum number of binding sites) and IC50 (concentration producing 50% reduction of the binding of the labelled ligand) were calculated by nonlinear fitting analysis computerized programs (Barlow 1983). When appropriate, pK_i ($-\log K_i$) values were obtained from IC50 values according to the equation $K_i = IC50/(1 + L/K_D)$ (Cheng & Prusoff 1973), where L is the concentration and K_D the equilibrium dissociation constant of the tritiated ligand used in the different binding assays.

Results are given as means ± s.e.m. for n experiments. Student's unpaired *t*-test was used for statistical analysis and $P < 0.05$ was considered as significant.

Results

Effects of muscarinic antagonists on acetylcholine and on contraction in tracheal strip

It was previously demonstrated that in the preparation used in this study the electrically-evoked outflow of ^3H reflects the neuronal release of newly synthesized acetylcholine and accordingly the prejunctional effects, whereas the stimulation-evoked contractile response reflects postjunctional effects (Kilbinger et al 1991).

Strips were stimulated with electrical field stimulation (EFS) three times (S_1, S_2 and S_3). In control experiments the ^3H outflow evoked by S_2 and S_3 was 0·78 ± 0·01 and 0·69 ± 0·02 (n = 8), respectively, of that induced by S_1 (5856 ± 693 Bq (g tissue)$^{-1}$). EFS also produced smooth muscle contraction. In control experiments the contractile response induced by S_1 (4·9 ± 0·5 g, n = 8) was not significantly different from that evoked by S_2 and S_3 ($P > 0.05$).

The antagonists were added cumulatively. The basal output of ^3H was not affected during the exposure to all the antagonists. Conversely, all the compounds produced a concentration-dependent increase of the electrically-evoked outflow of [^3H]acetylcholine. During EFS in the presence of telenzepine (0·001–1 μM), 4-DAMP (0·001–1 μM), *p*-F-HHSiD (10 nM–10 μM) and (±)-LG50643 (0·1 nM–1 μM) the enhancement in the overflow paralleled the reduction of the contractile response (Fig. 2). The behaviour observed for telenzepine (M_1-selective), 4-DAMP and *p*-F-HHSiD (M_3-selective) was similar to that described in previous studies (Kilbinger et al 1991; Ten Berge et al 1993). On the contrary, the M_2-selective antagonists methoctramine and gallamine displayed a different pattern. At low concentrations (30–100 nM) methoctramine caused a significant increase of the stimulated-induced contractile response ($P < 0.05$) and up to 10 μM it failed to reduce significantly the postjunctional response. Only at 100 μM was a significant inhibition (82% ± 8, n = 4) found. Similarly, during the exposure of gallamine (1–100 μM) the electrically-evoked contractile response was enhanced in a concentration-dependent manner. In a separate set of experiments a higher concentration (1 mM) did not cause any further enhancement; on the contrary, the electrically-evoked smooth muscle response was not affected in comparison with control experiments ($P > 0.05$, Fig. 3). During the exposure to 1 mM gallamine the

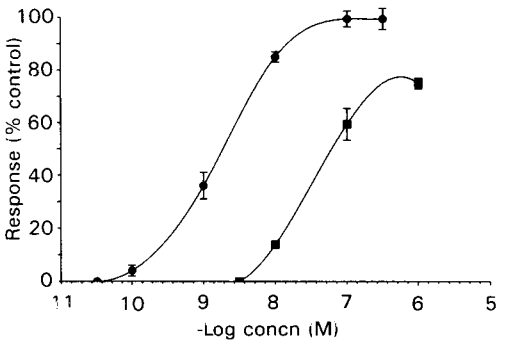

FIG. 2. Concentration-response curves of (±)-LG50643 for facilitation of [^3H]acetylcholine release (■) and inhibition of smooth muscle contraction (●) evoked by electrical stimulation in the guinea-pig epithelium-deprived trachea. The effect of the antagonist is given as percentage of the corresponding control value. Each point is the mean (s.e.m. shown by vertical lines) of 4–8 experiments.

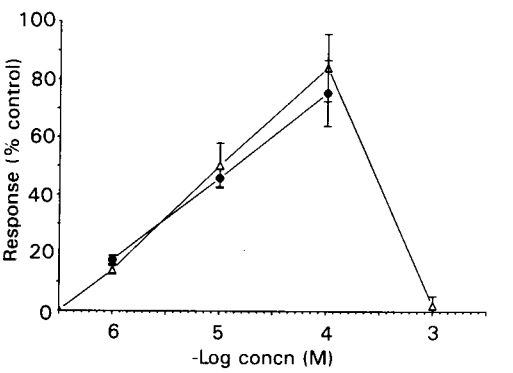

FIG. 3. Concentration-response curves of gallamine for facilitation of [^3H]acetylcholine release (●) and inhibition of smooth muscle contraction (△) evoked by electrical stimulation in the guinea-pig epithelium-deprived trachea. The effect of the antagonist is given as percentage of the corresponding control value. Each point is the mean (s.e.m. shown by vertical lines) of 4–6 experiments.

Table 2. Comparison between pre- and postjunctional potencies and postjunctional affinities of muscarinic antagonists in the guinea-pig isolated trachea.

Antagonist	$-\log EC50_{pre}$	$-\log EC50_{post}$	pA_2	$EC50_{pre}/EC50_{post}$
Telenzepine	7·98±0·19	7·84±0·10	7·9	0·72
Methoctramine	6·83±0·14	4·90±0·09**	6·1[a]	0·011
Gallamine	5·31±0·16	<3**	<4[a]	<0·005
4-DAMP	7·81±0·10	8·90±0·08*	9·1[a]	12·3
p-F-HHSiD	5·97±0·06	6·98±0·16*	7·1[b]	10·3
(±)-LG50643	7·47±0·05	8·90±0·15**	9·1	27

The $-\log EC50$ values (±s.e.m.) were obtained in 4–8 experiments. The table is based on studies in which pA_2 values were determined from a Schild plot with slope not different from unity. *$P<0·05$ **$P<0·01$ compared with corresponding prejunctional values. [a]Data from Eglen & Whiting (1988). [b]Datum from Eglen et al (1990).

Table 3. Antagonist affinities (pK_i) for muscarinic-receptor subtypes.

	Cerebral cortex (M_1)	Heart (M_2)	Salivary glands (M_3)	Selectivity	
				M_3/M_1	M_3/M_2
p-F-HHSiD	7·78	6·48**	7·98	1·6	30
4-DAMP	8·82	7·77*	8·98	1·4	16
LG50643	8·20	7·47*	8·64	2·7	15

The data are mean values determined in 3–6 experiments in rat tissues, each carried out in triplicate; s.e.m. did not exceed 0·1 log unit. Affinity values ($-\log K_i$) were measured by competition with [^{3}H]QNB binding, as explained in Materials and Methods. *$P<0·05$, **$P<0·01$ compared with the corresponding value at the M_3 site.

resting tension of the preparation was increased (0·86±0·06 g, n=3).

The potencies ($-\log EC50$) of the antagonists at pre- and postjunctional level are shown in Table 2.

Postjunctional affinity value

Telenzepine and (±)-LG50643, in a concentration range of 0·1 nM–1 μM, caused a parallel shift to the right of the concentration-response curve for carbachol without affecting the maximal contraction. The pA_2 value, determined from the Schild plot by regression analysis with a slope not different from unity, is reported in Table 2.

Affinity values for muscarinic binding sites

(±)-LG50643 was screened in comparison with M_3 muscarinic antagonists for muscarinic binding sites in three tissues of the rat (cerebral cortex, heart and salivary glands), representative for different muscarinic receptor subtypes (M_1, M_2 and M_3, respectively).

The pK_i values of the antagonists, resulting from 3 to 6 [^{3}H]QNB displacement curves, are given in Table 3.

Discussion

The anticholinergic properties of (±)-LG50643 in the guinea-pig trachea were tested by using an in-vitro technique in which the functional responses following pre- and postjunctional receptor blockade (facilitation of neurotransmitter release and inhibition of smooth muscle contraction, respectively) can be measured simultaneously. The comparison of pre- and postjunctional potencies ($-\log EC50$) of (±)-LG50643 indicates a clearcut (about 30-fold) selectivity of this compound for postjunctional receptors (Table 2). Such muscarinic receptors have been classified as M_3-type (Maclagan & Barnes 1989). In addition, the postjunctional affinity value ($pA_2=9·1$) is close to that found at the M_3 receptor in the ileal smooth muscle ($pA_2=8·92$, unpublished data).

Compared with 4-DAMP and *p*-F-HHSiD, M_3-subtype-preferring antagonists, (±)-LG50643 in functional experiments possesses a similar or higher potency at the M_3 receptor of the guinea-pig trachea (Table 2). Further, the $EC50_{pre}/EC50_{post}$ ratio indicates (±)-LG50643 is the most selective compound for this receptor.

In binding experiments, (±)-LG50643 binds with high but different affinity to muscarinic-receptor subtypes. The affinity constants (pK_i values) estimated at M_1, M_2 and M_3 sites in the reference tissues indicate a rank order of selectivity for this compound ($M_3>M_1>M_2$) similar to that found for 4-DAMP and *p*-F-HHSiD (Table 3). The results obtained in both functional and binding experiments indicate (±)-LG50643 is a novel M_3-subtype-preferring antagonist that can be used as a tool in muscarinic receptor characterization. In particular, as discussed above, the clear-cut selectivity of (±)-LG50643 for the postjunctional M_3 muscarinic receptor of the guinea-pig trachea might be of interest in the development of a new class of bronchodilator agents.

As far as the characterization of prejunctional muscarinic receptors is concerned, the pharmacological profiles of the subtype-preferring antagonists telenzepine, methoctramine, *p*-F-HHSiD and 4-DAMP confirm the heterogeneity of muscarinic receptors located at the two sides of the neuronal junction of the guinea-pig trachea (Kilbinger et al 1991). The ability shown by gallamine, a suggested M_2 antagonist, to discriminate (by about 200-fold) at pre- and postjunctional levels (Table 2) further supports such a heterogeneity. Recently, it has been reported that in this tissue, prejunctional autoreceptors might be similar to the M_4 site (Kilbinger et al 1993). Gallamine has also been proposed as a M_2/M_4 receptor-selective ligand (Michel & Whiting 1990). The data obtained in this study with gallamine supports such a suggestion.

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