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SPECIAL REPORT

4-DAMP analogues reveal heterogeneity of M_1 muscarinic receptors

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The muscarinic receptors responsible for two effects elicited by McN-A-343, i.e. the relaxation of the rat duodenum and the inhibition of the twitch contraction of rabbit vas deferens, were investigated by use of derivatives of 4-diphenyl acetoxy-N-methyl piperidine methobromide (4-DAMP). Both receptors had been previously identified as M_1 on the basis of the high affinity shown towards pirenzepine. Schild analysis of antagonism revealed that the affinities of 4-DAMP and three of its analogues in the rat duodenum were significantly different from those estimated in rabbit vas deferens. These data indicate that distinct receptor subtypes mediate duodenal relaxation and vas deferens inhibition of twitch contraction and suggest that receptors classified as M_1 by means of pirenzepine affinity constitute a heterogeneous population.

Introduction The classification of muscarinic receptors has long relied on the discriminative properties of the antagonist pirenzepine (Hirschowitz *et al.*, 1984). While receptors with low affinity for pirenzepine, originally defined as M_2, have subsequently proved to be heterogeneous and have been further characterized by means of newly developed selective antagonists (Levine *et al.*, 1988), the identification of the M_1 subtype is still based on pirenzepine recognition of receptor sites occurring with high affinity. Thus, in accordance with the high sensitivity shown towards pirenzepine blockade, two recently described neuronal responses have been attributed to stimulation of M_1 receptors. These are the relaxation of the isolated small intestine of the rat, due to muscarinic-mediated activation of a neural inhibitory pathway (Micheletti *et al.*, 1988) and the inhibition of the electrically-induced twitch contraction of the rabbit isolated vas deferens, occurring through prejunctional inhibitory muscarinic receptors (Eltze, 1988). Both responses are elicited by McN-A-343 and antagonized by pirenzepine with an affinity in the nanomolar range, consistent with interaction at M_1 sites.

To characterize further the receptor responsible for the above described effects, we studied the antagonism by 4-diphenyl acetoxy-N-methyl piperidine methobromide (4-DAMP) and some related compounds. Although commonly employed for its ability to discriminate between smooth muscle (M_3) and cardiac (M_2) receptors, 4-DAMP appears also endowed with some M_1 selectivity, in that it was shown to bind to M_1 receptors with high affinity (Berrie *et al.*, 1983) and to antagonize the M_1-mediated depolarization of the rat superior cervical ganglion with a pA_2 close to that found in the ileum (Brown *et al.*, 1980).

We now show that 4-DAMP and three of its derivatives antagonize the duodenal relaxation and the inhibition of twitch in the vas deferens with different affinities, indicating that a hitherto undescribed subtype is responsible for either of the two effects studied.

Methods Rats (male, Sprague Dawley, 200 g) were killed by cervical dislocation. Duodenum preparations were set up as described by Micheletti *et al.* (1988). Briefly, segments of duodenum of about 2 cm length were mounted in Tyrode solution (mM: NaCl 137, KCl 2.68, $CaCl_2$ 1.82, $NaHCO_3$ 5.9, $MgCl_2$ 1, NaH_2PO_4 0.42 and glucose 5.6) maintained at 37°C, under 1.5 g tension. Relaxation was induced by administering sequential concentrations of McN-A-343 at 15 min intervals.

Rabbits (male, New Zealand, 2.5–3.0 kg) were killed by cervical dislocation and exsanguinated. The epididymal portion of the vas deferens was mounted under 500 mg tension in the following solution (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, containing yohimbine 1 μM, and maintained at 32°C (Eltze, 1988). Preparations were continuously stimulated through platinum electrodes at 30 V, 0.5 ms, 0.05 Hz. McN-A-343 was added in cumulative concentrations.

In either preparation, isotonic contractions were recorded (Basile 7050 transducer). Antagonists were allowed 60 min equilibration period; each concentration was tested on a separate preparation. Affinity (expressed as $-\log K_B$) was estimated by Schild analysis, fitting linear regressions of individual experiments by the least squares method and verifying parallelism before calculating dose-ratios. Statistical comparison of Schild regressions was performed by parallel line bioassay (Finney, 1964), calculating potency ratios and confidence limits for $P < 0.05$ and $P < 0.01$. Significance was accepted at the $P < 0.05$ level.

All drugs used were synthesized in our laboratories: McN-A-343 (4-chlorophenylcarbamoyloxy-2-butynyltrimethylammonium chloride); pirenzepine; 4-DAMP (4-diphenylacetyoxy-N-methyl piperidine methobromide); α-methyl 4-DAMP (4-diphenyl-α-methylacetoxy-N-methylpiperidine methobromide); DA 6206 (1-iminoethyl)-4-piperidinyl α-methyl-α- phenylbenzeneacetate hydrochloride); DA 6264 (1-guanyl-4-piperidinyl α-methyl-α- phenylbenzeneacetate hydrochloride); DA 6265 (1-iminomethyl-4-piperidinyl α-methyl-α-phenylbenzeneacetate hydrochloride) (Eur. Pat. Appl. 309424).

Results McN-A-343 produced a relaxation of the rat duodenum and an inhibition of the twitch contraction of rabbit vas deferens with potencies comparable to those previously described (Micheletti *et al.*, 1988; Eltze, 1988): $-\log EC_{50}$ (M), 6.30 ± 0.05 and 5.86 ± 0.07, ($n = 15$, each), respectively.

The compounds investigated inhibited the effects of McN-A-343 in both preparations. The substances acted as competitive antagonists, producing parallel rightward displacements of McN-A-343 concentration-response curves and yielding slopes of Schild plots not significantly different from unity. The values found are reported in Table 1 and compared with those obtained with pirenzepine.

The potencies of 4-DAMP and its three amidino derivatives, DA 6206, DA 6264 and DA 6265, in inhibiting duodenal relaxation differed from those found in antagonizing the reduction of twitch contraction of rabbit vas deferens. In the case of 4-DAMP, affinity was higher for the vas deferens ($P < 0.05$), while its analogues displayed a tenfold greater affinity ($P < 0.01$) for the duodenal response. The compound α-methyl 4-DAMP did not distinguish between the two preparations. Affinity values in duodenum and vas deferens were

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Table 1 Affinity estimates and Schild slopes for 4-DAMP and its derivatives as antagonists at muscarinic receptors mediating rat duodenum relaxation and inhibition of rabbit vas deferens twitch contraction

	Rat duodenum		*Rabbit vas deferens*	
Compound	$-\log K_B$	Slope	$-\log K_B$	Slope
α-Methyl 4-DAMP	9.65 (0.14)	1.04 (0.18)	9.75 (0.30)	1.09 (0.24)
DA 6206	8.01 (0.19)	1.07 (0.19)	6.95 (0.09)**	1.14 (0.17)
DA 6264	8.40 (0.21)	0.91 (0.15)	7.22 (0.09)**	1.21 (0.12)
DA 6265	9.04 (0.10)	0.99 (0.09)	8.21 (0.16)**	0.83 (0.16)
4-DAMP	8.13 (0.26)	0.96 (0.21)	8.71 (0.10)*	1.23 (0.18)
Pirenzepine	8.09 (0.09)	1.14 (0.08)[a]	8.12 (0.05)	1.10 (0.12)

[a] Data from Micheletti *et al.* (1988).
* $P < 0.05$ and ** $P < 0.01$ in comparison with duodenum (parallel line bioassay).
Results from 9–15 replicates. In parentheses, s.e.mean.

very close and nearly identical to those reported for ileal and cardiac receptors (Barlow *et al.*, 1986, Table 1). Finally, estimates of pirenzepine affinity in either preparation were strictly comparable.

Discussion The present observations show that two muscarinic receptors, previously classified as belonging to the M_1 subtype, are in fact heterogeneous. These receptors mediate the relaxation of the rat isolated duodenum and the inhibition of the twitch contraction in rabbit vas deferens, the two effects being elicited by McN-A-343. While being antagonized by pirenzepine with high affinity, they display different sensitivities towards 4-DAMP and some related antagonists. As 4-DAMP and its amidino-derivatives show opposite selectivities, it seems unlikely that affinity measurements in the present studies were affected by the species used. The significantly different affinities found for these compounds thus indicate that distinct receptor subtypes sustain relaxation of duodenum and inhibition of rabbit vas deferens contraction.

A corollary to the present results is therefore that the selectivity of pirenzepine appears inadequate to define M_1 receptors, since the antagonist shows high affinity also for a non-M_1 subtype. This conclusion warrants some comment.

Of the five muscarinic receptor genes (m1–m5) described (Bonner *et al.*, 1987; 1988), the first three seem to correspond to M_1–M_3 receptors (Levine & Birdsall, 1989). The affinity pattern of pirenzepine for these subtypes is well-known. On the other hand, M_4 and M_5 receptors have not yet been distinctly identified and the knowledge available relates mainly to the localization and binding properties of the expressed m4 and m5 species. The present knowledge can be summarized as follows: (1) m1, m2, m3 and m4 receptors are coexpressed in the forebrain, including the cerebral cortex, a region widely employed for studies on M_1 receptors (Buckley *et al.*, 1989). (2) There are no known antagonists capable of specifically interacting with m4 and m5 receptors (Buckley *et al.*, 1989). (3) Pirenzepine is able to recognize m4 receptors with high affinity (Bonner *et al.*, 1987; Peralta *et al.*, 1987). This latter evidence was obtained during investigation of the properties of the individual subtypes expressed in transfected mammalian cells. Pirenzepine showed strikingly close affinities in displacing [^{3}H]-quinuclidinyl benzilate from m1 and m4 receptors in COS-7 cells (35 *vs.* 36 nM, Bonner *et al.*, 1987), and human embryonic kidney cells (less than 3 fold difference, Peralta *et al.*, 1987). A rather discrepant finding has been recently reported by Buckley *et al.* (1989), who showed that in CHO-K1 cells, pirenzepine was 6 fold less potent at m4 than m1 receptors; notably, affinity was not estimated because of the shallow Hill slope at m4 receptors. Plausible explanations for the low Hill number include negative cooperativity or post-translational modifications. Actually, the affinities of muscarinic receptor subtypes are known to be largely affected by membrane environment, since pirenzepine fails to discriminate between M_1 and M_2 subtypes following receptor solubilization and purification (Wang *et al.*, 1983; Berstein *et al.*, 1989), and the purified M_1 subtype recovers the original affinity upon interaction with factors present in membrane preparations (Berstein *et al.*, 1989).

In light of the above, the possibility cannot be excluded that previous studies may have classified m4 receptors as M_1 (Buckley *et al.*, 1989). It is then conceivable that an M_4 receptor subserves either of the functional responses studied here. Studies on cells that express the cloned individual subtypes should help to define the nature of the receptors present in duodenum and vas deferens and clarify the selectivity pattern of 4-DAMP and its analogues. These latter agents contain an amidine moiety of the formamidine (DA 6265), acetamidine (DA 6206) and guanidine (DA 6264) type. We have recently shown that the same amidine substitution affected the discriminative properties of hexocyclium derivatives, enhancing their selectivity for smooth muscle (M_3) receptors (Micheletti *et al.*, 1990). The improved selectivity of those antagonists seems to depend largely on a decrease of the M_2 affinity. In contrast, the M_2 and M_3 affinities of the 4-DAMP derivatives, DA 6265, DA 6206, DA 6264, are unchanged (data not shown).

It is hoped that the use of antagonists containing the amidine moiety will improve our knowledge of muscarinic receptor subtypes.

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