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The allosteric interaction of otenzepad (AF-DX 116) at muscarinic M_2 receptors in guinea pig atria

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Abstract

The effects of the muscarinic receptor antagonist, otenzepad, in combination with the competitive antagonists *N*-methylscopolamine, dexetimide and atropine, or the allosteric modulators, $C_7/3'$-phth, gallamine and alcuronium, were measured in the guinea pig electrically driven left atrium using the agonists, carbachol or acetylcholine. Otenzepad, in combination with $C_7/3'$-phth or gallamine, gave concentration-ratios close to additive and in agreement with theoretical model predictions for combination of two allosteric modulators acting at a common site. However, when otenzepad was combined with alcuronium, dexetimide or *N*-methylscopolamine, supra-additive effects were observed. For either competitive antagonist in combination with otenzepad, the degree of supra-additivity was more evident after 2-h equilibration than after 40 min. When otenzepad was combined with atropine, no supra-additivity was observed with carbachol as the agonist, but was evident with acetylcholine. Otenzepad was also unable to fully inhibit [^{3}H]*N*-methylscopolamine binding when the radioligand was employed at a concentration of $\sim 100 \times K_D$. It is concluded that the action of otenzepad involves an allosteric site and a number of possibilities are discussed for its location. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Allosteric; Muscarinic M_2 acetylcholine receptor; Otenzepad

1. Introduction

Otenzepad (AF-DX 116) exhibits an affinity for muscarinic M_2 receptors that is approximately 5–10-fold higher than that for other muscarinic receptor subtypes (Caulfield, 1993). It has been proposed as a treatment for sinus bradycardia since effects on cardiac rate may be obtained without inhibition of salivary flow or accommodation in the eye (Pitschner et al., 1989a,b; Schultz et al., 1991).

The nature of the interaction of otenzepad with muscarinic acetylcholine receptors has engendered a degree of controversy with some investigators concluding that otenzepad acted as a competitive antagonist (Hammer et al., 1986; Giachetti et al., 1986; Micheletti et al., 1987; Del Tacca et al., 1990) while others have found evidence for an allosteric interaction (Roffel et al., 1989; Lee and El-Fakahany, 1990; Pedder et al., 1991). Hammer et al. (1986) concluded that otenzepad was interacting in a competitive manner, rather than allosterically, since it completely inhibited binding of high as well as low concentrations of [^{3}H]*N*-methylscopolamine at the cardiac muscarinic M_2 receptor and did not affect the dissociation rate of [^{3}H]*N*-methylscopolamine in the presence of excess unlabelled *N*-methylscopolamine. Schild analysis of the inhibitory effect of otenzepad on muscarinic M_2 receptors in isolated guinea pig atria was also consistent with competitive antagonism, the resulting slope of the plot being not significantly different from unity (Giachetti et al., 1986; Del Tacca et al., 1990). In contrast, the action of otenzepad in bovine cardiac left ventricular muscle and tracheal smooth muscle (Roffel et al., 1989) and in rat heart membranes (Lee and El-Fakahany, 1991) suggested it acted in an allosteric fashion and that methoctramine, gallamine and otenzepad interacted at the same secondary binding site on the muscarinic receptor (Roffel et al., 1989).

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Some investigators have proposed that otenzepad has a more complex 'dual mode' of interaction with the muscarinic receptor; that is, both competitive and allosteric inhibition may be present (Kunysz et al., 1988; Lee and El-Fakahany, 1991; Boselli and Grana, 1995).

To provide more definitive evidence of the allosteric action of otenzepad on the muscarinic M_2 receptor, its interactions with the allosteric modulators, heptane-1,7-bis(dimethyl-3′-phthalimidopropylammonium bromide) ($C_7/3'$-phth), gallamine and alcuronium, were investigated in functional combination experiments. For comparison, similar experiments were conducted with competitive antagonists, *N*-methylscopolamine, dexetimide and atropine, and radioligand binding studies with $[^3H]$*N*-methylscopolamine were also undertaken. In this study, we provide evidence that otenzepad acts competitively with two other allosteric modulators as well as allosterically with three competitive antagonists, consistent with an action of otenzepad at an allosteric site on muscarinic M_2 receptors.

2. Materials and methods

2.1. Materials

Drugs used were $[^3H]$*N*-methylscopolamine chloride (Amersham, Amersham, UK), heptane-1,7-bis(dimethyl-3′-phthalimidopropylammonium bromide) (Institute of Drug Technology, Boronia, Australia), atropine sulphate, acetylcholine chloride, carbamoylcholine chloride (carbachol), dyflos, gallamine triethiodide, *N*-methylscopolamine bromide (Sigma, St. Louis, MO), dexetimide hydrochloride (gift; Janssen, Pharmaceuticals, Beerse, Belgium), otenzepad (AF-DX 116) (gift; Dr. Karl Thomae, Biberach, Germany) and alcuronium chloride (gift; Hoffman–La Roche, Basle, Switzerland).

2.2. Organ bath preparations

Guinea pigs of either sex were killed by cervical dislocation followed by exsanguination, and their hearts were rapidly removed and placed in ice-cold Krebs' solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7 and $CaCl_2$ 2.2. The left atrium was dissected out, attached to a tissue hook on the end of an electrode assembly and placed in a 20-ml organ bath containing Krebs buffer at 37°C, bubbled with a mixture of 95% O_2 and 5% CO_2. A Grass force-displacement transducer (FT.03C), connected to a Grass polygraph (Model 79D), was used to record the responses. The atrium was electrically driven by a Grass S48 stimulator (3 Hz, 10 ms, 15 V). The tissue was allowed to equilibrate for at least 20 min under a resting tension of 1 g before exposure to an agonist. In experiments where acetylcholine was the agonist, the tissue was incubated initially with the irreversible cholinesterase inhibitor dyflos (10 μM) for 20 min, followed by washing for 20 min, prior to agonist exposure.

2.3. Experiments using competitive antagonists or allosteric modulators

A contact time of 1 min with the tissue was employed for each concentration of agonist (acetylcholine or carbachol). The geometric mean EC_{50} value (95% confidence limits, n) for acetylcholine was 17.2 nM (11.4–26.2; 10) and for carbachol, 0.17 μM (0.12–0.23; 10). The tissue was washed twice with fresh Krebs solution following addition of each concentration of agonist, with 5-min periods allowed between additions. A minimum of five concentrations of the agonist were used to construct a control concentration–response curve, ranging from 20% to 80% of maximal inhibition, with negative, inotropic responses to any one concentration being duplicated over a period of 20–30 min.

The agonist concentration–response curve was then re-established after incubation of the atrium with different concentrations of either the competitive antagonists, *N*-methylscopolamine, dexetimide and atropine, or the allosteric modulators, $C_7/3'$-phth, gallamine or alcuronium, with responses to agonists being obtained in duplicate, as described above. The initial equilibration time for the inhibitors was 40 min except in the case of dexetimide and *N*-methylscopolamine (10 nM), where 90 min was employed, with washing and immediate replacement of inhibitor every 20 min. All the inhibitors caused parallel displacement of the agonist concentration–response curves with no suppression of the maximal response.

2.4. Experiments with combinations of otenzepad and inhibitors

After re-establishment of a concentration–response curve in the presence of one concentration of inhibitor, followed by washing of the tissue, a second inhibitor was added together with the initial inhibitor and an additional 40-min period of equilibration with the tissue was undertaken, after which a third concentration–response curve for the agonist was determined. In some experiments, the incubation period with both inhibitors present in the tissue bath was extended up to 240 min, with washing and immediate replacement every 30 min. In the same fashion as for the other curves, responses to the agonist were duplicated over a period of 20–30 min.

In other experiments, the inhibitors were added in reverse sequence to check if the order of addition affected the concentration-ratio obtained with the various combinations of inhibitors.

2.5. Radioligand binding studies

Guinea pigs of either sex were killed by exsanguination and their hearts were rapidly removed and placed in ice-cold phosphate buffer (50 mM NaH_2PO_4, pH 7.4). The left and right atria were then separated from the ventricular tissue, blotted dry, weighed, and minced finely with scissors. Phosphate buffer (15 volumes) was added and the resulting suspension was homogenised in an Ultra Turrax (0.75 × maximum speed) for 2 × 30-s bursts, with a 30-s period of cooling on ice between homogenisation. The homogenate was then centrifuged for 10 min at 1000 × g, after which the supernatant was collected and used in the radioligand binding experiments. The inhibition binding experiments were conducted in phosphate buffer and involved incubating 200-μl aliquots of atrial homogenate with increasing concentrations of otenzepad (1 nM–500 μM), in the presence of a fixed concentration of $[^3H]$*N*-methylscopolamine. Each experiment was performed on the basis of a two-curve assay, where the fixed concentration of $[^3H]$*N*-methylscopolamine was 0.3 nM ($\sim K_D$) for the first inhibition curve and 30 nM ($\sim 100 \times K_D$) for the second inhibition curve. The assays were conducted in 1-ml final volume/tube and incubated for 1 h at 37°C before termination by vacuum filtration through Whatman GF/B filters (pre-soaked in phosphate buffer containing 10-μM atropine and 0.5% polyethylenimine) using a Brandel cell harvester. Filters were washed three times with 4-ml aliquots of ice-cold phosphate buffer and counted in plastic vials, containing 5 ml of Filter Count (Packard), by a liquid scintillation analyser. Non-specific binding was determined using atropine (0.3 μM) and in all cases, total binding was < 10% of added radioligand.

2.6. Data analysis

Using the program PRISM 2.01 (GraphPad Software, San Diego, CA), the EC_{50} values for agonists (concentration producing 50% inhibition of atrial contractility) from the concentration–response curves were determined by fitting the data to an equation of the following form:

$$E = \frac{100[A]}{(EC_{50} + [A])} \qquad (1)$$

where E is the effect, expressed as % inhibition of atrial contractility, and [A] is the concentration of agonist. Concentration-ratios were calculated as the ratio of the EC_{50} obtained in the presence of inhibitor(s) to that obtained in the absence of inhibitor(s), and are expressed as a geometric mean together with 95% confidence limits.

In experiments where carbachol was the agonist, the concentration-ratio obtained experimentally from the concentration–response curve, in the presence of *N*-methylscopolamine and otenzepad was compared to a predicted concentration-ratio for combination of a competitive antagonist and an allosteric modulator. The equation for the concentration-ratio under these conditions is given by the following expression (Christopoulos and Mitchelson, 1994);

$$CR_{BZ} = CR_Z \left[1 + (CR_B - 1) \left(\frac{\frac{[Z]}{\alpha' K_Z} + 1}{\frac{[Z]}{K_Z} + 1} \right) \right] \qquad (2)$$

where CR_{BZ}, CR_Z and CR_B are concentration-ratios for the combination of competitive antagonist plus allosteric modulator, the allosteric modulator (Z) alone and competitive antagonist (B) alone, respectively; α' is the heterotropic co-operativity factor for the interaction between Z and B; K_Z is the modulator-receptor equilibrium dissociation constant.

For experiments with two allosteric modulators (Y and Z) in combination, the concentration-ratio obtained was compared with that predicted from a model where an

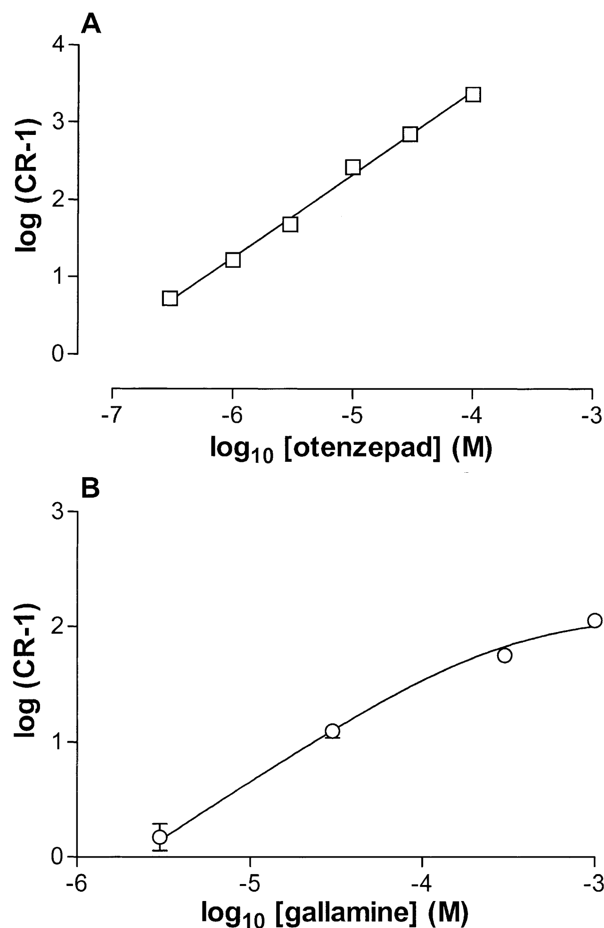

Fig. 1. (A) Schild plot for otenzepad versus carbachol. Each point is the mean of at least four experiments. Where not shown, the error bars lie within the dimensions of the symbol. (B) Schild plot for gallamine versus acetylcholine. Each point represents the mean of three experiments. Where not shown, the error bars lie within the dimensions of the symbol.

agonist interacts with two allosteric modulators that compete for a common site (Lanzafame et al., 1997). The equation for the predicted combination concentration-ratio (CR_{YZ}) is given by:

$$CR_{YZ} = \frac{1 + \frac{\beta(CR_Y - 1)}{(\beta - CR_Y)} + \frac{\alpha(CR_Z - 1)}{(\alpha - CR_Z)}}{1 + \frac{(CR_Y - 1)}{(\beta - CR_Y)} + \frac{(CR_Z - 1)}{(\alpha - CR_Z)}} \quad (3)$$

where α and β are the heterotropic co-operativity factors between the agonist and Z or Y, respectively.

In experiments assessing the functional interaction between gallamine and acetylcholine, estimates of the modulator-receptor dissociation constant (K_Z) and co-operativity factor (α) for the interaction were obtained from the relationship;

$$CR_Z - 1 = \frac{(\alpha - 1)}{\left(\frac{\alpha K_Z}{[Z]} + 1\right)} \quad (4)$$

where CR_Z is the concentration-ratio produced by a concentration [Z] of gallamine (Ehlert, 1988).

For the radioligand binding experiments, the program Scientist for Windows 2.01 (Micromath Scientific Software, Salt Lake City, UT) was used to simultaneously analyse the results of each two-curve assay, in order to determine the co-operativity factor (α') for the interaction between otenzepad and [^{3}H]*N*-methylscopolamine. This was achieved using the relationship between fractional occupancy $(Y)/(Y_0)$ of [^{3}H]*N*-methylscopolamine (A) in the presence of the modulator, otenzepad (Z) according to Ehlert (1988).

$$\frac{Y}{Y_0} = \frac{[A]}{[A] + K_A\left[\frac{K_Z + [Z]}{K_Z + \frac{[Z]}{\alpha'}}\right]}. \quad (5)$$

Statistical significance was determined using Student's *t*-test; values of $P < 0.05$ were considered significant.

3. Results

3.1. Otenzepad versus carbachol

Otenzepad produced a linear Schild plot over a wide concentration range (0.3 to 100 μM) giving concentration-ratios up to 2400 (Fig. 1A). The slope of the plot was 1.07 ± 0.03 (40 data points; six concentrations) and was not significantly different from unity ($P > 0.05$). The pA_2 value was 7.16. Fitting a line with unit slope gave a K_B of 51.2 nM (95% confidence limits; 41.8–62.5 nM).

Control experiments with carbachol as the agonist repeated at 90, 240 and 320 min showed that the EC_{50} for the agonist did not change significantly ($P < 0.05$) throughout the experiment (data not shown). Similar findings were obtained when acetylcholine was used as the agonist.

Table 1
Concentration-ratios produced by otenzepad alone (CR_1) or in combination with an allosteric modulator

Agonist	Otenzepad (μM)	CR_1^a	Allosteric modulator (μM)	CR_2^b	Experimental combination CR[c]		Predicted combination CR of ternary complex[d]
					CR_{40}	CR_{120}	
Carbachol	3	50 (41–61; 4)	$C_7/3'$-phth (10)	112 (81–135; 5)	139 (76–255; 4)	139 (82–236; 4)	154 (146–163; 4)
	10	323 (181–578; 4)	$C_7/3'$-phth (10)	112 (81–135; 5)	390 (290–525; 4)	403 (273–594; 4)	397 (257–614; 4)
	10	284 (107–757; 4)	$C_7/3'$-phth (30)	321 (270–381; 4)	627 (483–1439; 4)	621 (267–1447; 4)	522 (358–760; 4
	1	17 (8–35; 3)	alcuronium (10)	5 (4–7; 15)	49 (39–62; 3)	88 (50–156; 3)	21 (12–37; 3)
	10	256 (145–451; 4)	alcuronium (10)	5 (4–7; 15)	392 (221–693; 4)	559 (313–998; 4)	259 (148–452; 4)
Acetylcholine	1	15 (14–15; 4)	$C_7/3'$-phth (10)	112 (81–135; 5)	123 (99–153; 4)	130 (105–162; 4)	124 (123–125; 4)
	10	329 (198–546; 4)	gallamine (100)	22 (18–27; 4)	291 (128–659; 4)	285 (141–578; 4	301 (186–485; 4)

Concentration-ratio is represented by CR.
[a]Geometric mean CR (95% confidence limits; number of experiments).
[b]Determined in separate experiments where the modulator was used alone as the inhibitor.
[c]CR_{40} and CR_{120}: subscript represents equilibration time (min) with the combination of inhibitors.
[d]Predicted combination CR (geometric mean value) if both inhibitors were acting allosterically, given by Eq. (3) (see Section 2), using an α value for otenzepad of 3000 with either agonist. The value of β used for $C_7/3'$-phth was 1063 with carbachol and 1679 with acetylcholine (see Lanzafame et al., 1996); for alcuronium with carbachol, 1000 (see Lanzafame et al., 1997) and for gallamine with acetylcholine, 130.

Table 2
Concentration-ratios produced by a competitive antagonist alone (CR_1) or in combination with otenzepad

Agonist	Competitive antagonist (nM)	CR_1^a	Otenzepad (μM)	CR_2^b	Experimental combination CR[c]		Predicted combination CR[d] ($CR_1 + CR_2 - 1$)	Ratio	
					CR_{40}	CR_{120}		$(CR_{40})/(CR_1 + CR_2 - 1)$	$(CR_{120})/(CR_1 + CR_2 - 1)$
Carbachol	Dexetimide (10)	56 (30.5–101; 4)	10	266 (219–324; 17)	382 (240–609; 4)	467 (260–839; 4)	324 (289–362; 4)	1.18	1.44
	Dexetimide (30)	271 (197–372; 4)	10	266 (219–324; 17)	1123 (671–1882; 4)	1258 (876–1806; 4)	538 (461–629; 4)	2.09	2.34
	Atropine (200)	263 (209–331; 6)	10	266 (219–324; 17)	484 (366–641; 6)	614 (388–973; 6)	531 (474–595; 6)	0.91	1.16
Acetylcholine	Atropine (200)	255 (195–333; 3)	10	329 (198–546; 4)	568 (338–957; 3)	888 (688–1147; 3)	583 (519–656; 3)	0.97	1.52

Concentration-ratio is represented by CR.
[a] Geometric mean CR (95% confidence limits; number of experiments).
[b] Determined in separate experiments where otenzepad was used alone as the inhibitor.
[c] CR_{40} and CR_{120}: subscript represents equilibration time (min) with the combination of inhibitors.
[d] Predicted combination CR (geometric mean value) if both inhibitors were acting competitively, given by ($CR_1 + CR_2 - 1$) (Paton and Rang, 1965).

Table 3
Concentration-ratios produced by the combination of the two inhibitors, otenzepad and *N*-methylscopolamine using carbachol as agonist

First inhibitor (μM)	CR_1^a	Second inhibitor (μM)	CR_2^b	Experimental combination CR[c]				Predicted combination CR[d]	
				CR_{40}	CR_{120}	CR_{180}	CR_{240}	Two competitive	Allosteric + competitive
Otenzepad (10)	223 (185–269; 5)	NMS (0.01)	186 (83–415; 4)	447 (334–598; 5)	609 (301–1229; 4)	543[e] (408–723; 3)	544[e] (376–776; 3)	409 (369–453; 5)	456 (378–550; 5)
NMS (0.01)	186 (83–415; 4)	Otenzepad (10)	266 (219–324; 17)	674 (371–1101; 4)	–	767[e] (535–1101; 4)	781[e] (539–1133; 4)	461 (332–640; 4)	558 (371–839; 4)
NMS (0.03)	248 (221–277; 4)	Otenzepad (10)	266 (219–324; 17)	636[e] (518–782; 4)	900[e,f] (782–1036; 4)	–	–	513 (486–541; 4)	638 (782–1036; 4)

Concentration ratio is represented by CR and *N*-methylscopolamine is represented by NMS.

[a] Geometric mean CR for first inhibitor (95% confidence limits; number of experiments).

[b] Determined in separate experiments where the second inhibitor was used alone.

[c] CR_{40-240}: subscript represents equilibration time (min) with the combination of inhibitors.

[d] Two competitive: predicted CR for combination of two competitive inhibitors, allosteric + competitive : predicted CR for combination of an allosteric modulator with a competitive antagonist, given by Eq. (2) (Section 2) using the α' value of 522 for the interaction between otenzepad and *N*-methylscopolamine.

[e] Significantly different ($P < 0.05$) from CR predicted for the combination of two competitive antagonists.

[f] Significantly different ($P < 0.05$) from CR predicted for the combination of an allosteric modulator and a competitive antagonist.

3.2. Gallamine versus acetylcholine

Gallamine produced a curvilinear Schild plot as expected for a negative allosteric modulator (Clark and Mitchelson, 1976; Ehlert, 1988) as shown in Fig. 1B. The K_Z value for gallamine was estimated as 2.1 μM (1.3–3.4 μM; $n = 3$) with a co-operativity factor (α) of 130 ± 22 using Eq. (4).

3.3. Otenzepad in combination with allosteric modulators

Experiments involving the combination of otenzepad (1, 3 and 10 μM) with $C_7/3'$-phth (10 or 30 μM), using carbachol as agonist, gave concentration-ratios that were close to additive (Table 1). No significant changes were observed, when the incubation period was extended to 120 min with the two inhibitors. Otenzepad (1 μM) in combination with $C_7/3'$-phth (10 μM) using acetylcholine as agonist, also gave close to additive concentration-ratios (Table 1). This phenomenon was consistent with concentration-ratios predicted for a ternary complex model involving two allosteric modulators competing for the same binding site (Table 1).

Otenzepad (10 μM) in combination with gallamine (100 μM), using acetylcholine as agonist, gave concentration-ratios that were under-additive but comparable to the combination concentration-ratio predicted by a model based on a ternary complex in which otenzepad and gallamine were competing for a common modulatory site (Table 1).

When combined with alcuronium (10 μM), otenzepad (1 μM) produced 2.3- to 4.2-fold supra-additive inhibition of responses to carbachol after 40- and 120-min incubation periods, respectively (Table 1). When a higher concentration of otenzepad (10 μM) was combined with the same concentration of alcuronium, the resulting combination concentration-ratio exhibited a lower degree of supra-additivity, 1.5- to 2.1-fold, after 40- and 120-min incubation times, respectively (Table 1). These findings suggested that otenzepad was not acting solely at the allosteric site recognised by alcuronium. Combination studies involving otenzepad and various competitive antagonists were then conducted to further elucidate the action of this inhibitor.

3.4. Otenzepad in combination with competitive antagonists

When the competitive antagonist dexetimide (30 nM) was combined with otenzepad (10 μM), the combination concentration-ratios produced were significantly ($P < 0.05$) supra-additive after both 40- and 120-min incubation times (Table 2). With a lower concentration of dexetimide (10 nM), no significant ($P > 0.05$) supra-additivity was observed (Table 2).

Results obtained for the combination of atropine (0.2 μM) with otenzepad (10 μM), using acetylcholine as agonist, showed that after 120-min incubation with both inhibitors 1.5-fold supra-additivity was observed ($P < 0.05$; Table 2). When carbachol was employed as the agonist, the combination of otenzepad and atropine gave additive inhibition (Table 2).

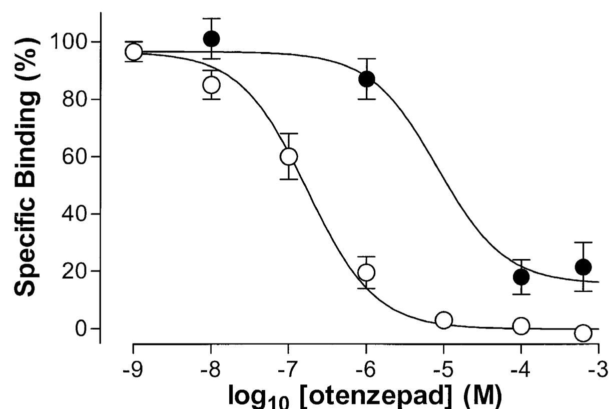

Fig. 2. Inhibition of [^{3}H]*N*-methylscopolamine binding 0.3 nM (○) and 30 nM (●) by increasing concentrations of otenzepad. Experiments were carried out using guinea pig atria in 50-mM phosphate buffer (pH 7.4) at 37°C. Each point represents the mean ± S.E.M of three to five experiments. Lines represent the mean best fit by simultaneous non-linear regression (see Section 2). Where not shown, the error bars lie within the dimensions of the symbol.

Combination studies with otenzepad and *N*-methylscopolamine showed a small but significant ($P < 0.05$) degree of supra-additivity (1.3- to 1.8-fold) after prolonged incubation with the two inhibitors (Table 3) and the sequential order of addition of the two inhibitors did not alter the degree of supra-additivity obtained. The concentration-ratios obtained were in general agreement with those predicted for a ternary complex between an agonist, competitive antagonist and allosteric modulator.

3.5. [^{3}H]N-methylscopolamine inhibition studies

As shown in Fig. 2, otenzepad inhibited the binding of [^{3}H]*N*-methylscopolamine (0.3 nM, $\sim K_D$) in a concentration-dependent manner. However, when a $\sim 100 \times K_D$-concentration of the radioligand (30 nM) was used, $17 \pm 4\%$ of [^{3}H]*N*-methylscopolamine binding was not inhibited by otenzepad, indicative of an allosteric interaction. Simultaneous analysis of the data for the two concentrations of [^{3}H]*N*-methylscopolamine according to Eq. (5) gave a K_Z of 72.4 nM (52.5–100.0 nM) and an α' of 522 ± 80.

4. Discussion

Otenzepad, over a 300-fold concentration range (0.3–100 μM), gave a linear Schild plot for inhibition of the response to carbachol. A linear plot was also obtained for otenzepad (1–100 μM) using acetylcholine as agonist (data not shown). While this may be taken as presumptive evidence for a competitive mode of interaction with the

agonists, it is not incompatible with otenzepad being a negative allosteric modulator with a high degree of negative co-operativity. Such a possibility certainly appears necessary to explain the findings obtained with otenzepad in combination with competitive antagonists.

Using dexetimide or atropine (with acetylcholine as agonist), otenzepad produced significant supra-additivity in combination, supporting a modulatory role for otenzepad via an action at an allosteric site. This was not as evident using *N*-methylscopolamine. With this latter antagonist, it was necessary to incubate the tissue with *N*-methylscopolamine and otenzepad for at least 2 h, and even then the degree of supra-additivity was small. However, clearer evidence in support of an allosteric action for otenzepad with *N*-methylscopolamine was obtained in the radioligand binding experiments. The binding of high concentrations of $[^3H]$*N*-methylscopolamine ($\sim 100 \times K_D$) could not be fully inhibited by high concentrations of otenzepad. Such an effect would not occur with two competitive antagonists; an increase in the concentration of one competitive antagonist should completely inhibit the binding of the other antagonist. The estimated α' value of 522, for the interaction of otenzepad and $[^3H]$*N*-methylscopolamine at the muscarinic M_2 receptor was used to predict the shift of the concentration–response curve to carbachol when *N*-methylscopolamine was combined with otenzepad. The predicted concentration-ratios obtained were within 70–85% of the experimental values obtained after equilibration times of ≥ 120 min (Table 3) and thus in good agreement. In contrast, the predicted values for two competitive antagonists were only within 55–75% of the experimental concentration-ratios.

A number of investigators have previously concluded that otenzepad acted allosterically (Roffel et al., 1989; Lee and El-Fakahany, 1990, 1991) or differently from competitive antagonists (Pedder et al., 1991). Other investigators have concluded that otenzepad acted as a competitive antagonist. One reason for this latter conclusion is that insufficient time may have been allowed for equilibration to occur, given the current realisation that allosteric modulators may significantly retard the rate of equilibration of orthosteric ligands with the receptor (Tucek and Proška, 1995). As has been noted in this study, the use of *N*-methylscopolamine and otenzepad in combination appeared to require a long period of equilibration to allow the small degrees of supra-additivity to become evident. Use of a 40-min period before retesting the shift of an agonist, concentration–response curve was not sufficient. Drug disposition on addition of a second drug may be a slow process, given that alternative binding sites in atria may influence equilibration time (Gray et al., 1976; Lüllmann et al., 1988). It is possible that similar times were employed by others who failed to detect evidence of allosteric modulation. Another reason is that otenzepad exhibits such high negative co-operativity that the interaction becomes difficult to differentiate from competition (Ehlert, 1988). For example, Hammer et al. (1986) specifically looked for evidence of allosterism and compared otenzepad with gallamine, a known allosteric modulator, at muscarinic M_2 receptors. While gallamine failed to fully inhibit $30 \times K_D$ of $[^3H]$*N*-methylscopolamine, otenzepad was effective. However, as has been demonstrated in the current study, the use of otenzepad with $100 \times K_D$ of $[^3H]$*N*-methylscopolamine did reveal evidence of allosterism. The difference was presumably due to the higher degree of negative co-operativity exhibited by otenzepad compared to that of gallamine.

Further evidence supportive of an allosteric site for otenzepad was provided by the combination of otenzepad with $C_7/3'$-phth, a known negative allosteric modulator (Lanzafame et al., 1996, 1997). Combination of these two compounds gave rise to a shift of the carbachol concentration–response curve that was not supra-additive (Table 1). This finding was in contrast to previous results with a number of competitive antagonists in combination with $C_7/3'$-phth (Mitchelson, 1975; Lüllmann et al., 1969; Lanzafame et al., 1997) and provided evidence in favour of a common site of action for both $C_7/3'$-phth and otenzepad. The combined concentration-ratio obtained was predictable on the basis that otenzepad and $C_7/3'$-phth were acting at a common modulatory allosteric site, with both inhibitors exhibiting high degrees of negative co-operativity against carbachol, the latter acting at the orthosteric site. Values of $\alpha = 1063$ for $C_7/3'$-phth versus carbachol (Lanzafame et al., 1996) and $\beta \geq 3000$ for otenzepad, based on the lack of curvature in the Schild plot for concentration-ratios up to 2400, were used to derive the predicted values (Table 1). Further evidence of allosterism was obtained using gallamine in combination with otenzepad and acetylcholine, as the combination gave concentration-ratios that were in agreement with both inhibitors being allosteric modulators acting at a common site. Previously, it had been shown that gallamine in combination with competitive antagonists, using carbachol as agonist, produced concentration-ratios that are similar to those expected for two competitive antagonists, whereas with acetylcholine as the agonist the combination concentration-ratios are significantly less than expected (Clark and Mitchelson, 1976).

In contrast to the findings with $C_7/3'$-phth or with gallamine, combination of alcuronium and otenzepad gave supra-additive concentration-ratios suggestive of alcuronium and otenzepad acting at different sites. If this were the case then alcuronium would need to bind to a different site to that of gallamine and $C_7/3'$-phth. However, Tucek and Proška (1995) provided evidence from binding studies with $[^3H]$*N*-methylscopolamine that alcuronium and gallamine behaved as predicted for two allosteric modulators acting at a common site and there is evidence from functional studies with alcuronium in combination with gallamine or $C_7/3'$-phth that the three allosteric modulators acted at a common site (Lanzafame et al., 1997).

One possibility to resolve the paradox is that otenzepad occupies a site that is different to the three allosteric modulators, alcuronium, gallamine and $C_7/3'$-phth and that occupancy of this site modulates binding of agonists at the orthosteric site to a similar extent to the modulation of gallamine or $C_7/3'$-phth whereas alcuronium is modulated to a lesser extent. One advantage of this scheme is that the ability of otenzepad to cause such a large degree of negative co-operativity may be more readily accommodated since none of the other allosteric modulators acting at the common site have such pronounced effects on competitive antagonist binding at the orthosteric site. Evidence in favour of a second allosteric site on muscarinic receptors has recently been presented for M_1 (Lazareno et al., 2000) and M_2 (Tränkle and Mohr, 1997) subtypes.

Tränkle et al. (1998) have offered another explanation for their findings with AF-DX 384 and W84 (congeners of otenzepad and $C_7/3'$-phth, respectively). They concluded that AF-DX 384 could attach to both the orthosteric site and the allosteric site occupied by W84 and that the attachment of AF-DX 384 may vary depending on which other ligand is present.

In conclusion, the evidence obtained in this study using otenzepad in combination with allosteric modulators or competitive antagonists suggests that otenzepad binds to an allosteric site at the muscarinic M_2 receptor. Further work is needed to resolve the conflicting findings that suggest a number of alternative possibilities about whether the site is similar to or overlaps that occupied by the other allosteric modulators and orthosteric ligands studied, or is it a distinct site from both of these sites on the muscarinic receptor. While the suggestion that otenzepad may bind to an allosteric site is not new the findings that otenzepad appears to act competitively with two other allosteric modulators, gallamine and $C_7/3'$-phth as well as allosterically with three competitive antagonists, atropine, dexetimide and *N*-methylscopolamine provides further new evidence in support of such an action.

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References

Boselli, C., Grana, E., 1995. Mode of antagonism of methoctramine, AF-DX 116 and hexahydrosiladifenidol in guinea-pig left atrium and ileum: comparison of Schild and resultant analysis. J. Auton. Pharmacol. 15, 115–127.

Caulfield, M.P., 1993. Muscarinic receptors—characterization, coupling and function. Pharmacol. Ther. 58, 319–379.

Christopoulos, A., Mitchelson, F., 1994. Assessment of the allosteric interactions of the bisquaternary heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl)-ammonium bromide at M_1 and M_2 muscarine receptors. Mol. Pharmacol. 46, 105–114.

Clark, A.L., Mitchelson, F., 1976. Inhibitory effect of gallamine on muscarinic receptors. Br. J. Pharmacol. 58, 323–331.

Del Tacca, M., Danesi, R., Blandizzi, C., Bernardini, M.C., 1990. Differential affinities of AF-DX 116, atropine and pirenzepine for muscarinic receptors of guinea pig gastric fundus, atria and urinary bladder: might atropine distinguish among muscarinic receptor subtypes? Pharmacology 40, 241–249.

Ehlert, F.J., 1988. Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. Mol. Pharmacol. 33, 187–194.

Giachetti, A., Micheletti, R., Montagna, E., 1986. Cardioselective profile of AF-DX 116, a muscarine M_2 receptor antagonist. Life Sci. 38, 1663–1672.

Gray, J.A., Lüllmann, H., Mitchelson, F., Reil, G.-H., 1976. Stereoselective binding in cardiac tissue of the enantiomers of benzetimide, an antimuscarinic drug. Br. J. Pharmacol. 56, 485–490.

Hammer, R., Giraldo, E., Schiavi, G.B., Monferini, E., Ladinsky, H., 1986. 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.

Kunysz, E.L., Michel, A.D., Whiting, R.L., 1988. Functional and direct binding studies using subtype selective muscarinic receptor antagonists. Br. J. Pharmacol. 93, 491–500.

Lanzafame, A., Christopoulos, A., Mitchelson, F., 1996. Interactions of agonists with an allosteric antagonist at muscarinic acetylcholine M_2 receptors. Eur. J. Pharmacol. 316, 27–32.

Lanzafame, A., Christopoulos, A., Mitchelson, F., 1997. Three allosteric modulators act at a common site, distinct from that of competitive antagonists, at muscarinic acetylcholine M_2 receptors. J. Pharmacol. Exp. Ther. 282, 278–285.

Lazareno, S., Popham, A., Birdsall, N.J.M., 2000. Allosteric interactions of staurosporine and other indolocarbazoles with *N*-[methyl-^{3}H]scopolamine and acetylcholine at muscarinic receptor subtypes: Identification of a second allosteric site. Mol. Pharmacol. 58, 194–207.

Lee, N.H., El-Fakahany, E.E., 1990. The allosteric binding profile of himbacine: a comparison with other cardioselective muscarinic antagonists. Eur. J. Pharmacol. 179, 225–229.

Lee, N.H., El-Fakahany, E.E., 1991. Allosteric interactions at the m1, m2 and m3 muscarinic receptor subtypes. J. Pharmacol. Exp. Ther. 256, 468–479.

Lüllmann, H., Ohnesorge, F., Schauwecker, G., Wassermann, O., 1969. Inhibition of the actions of carbachol and DFP on guinea pig isolated left atria by alkane bis-ammonium compounds. Eur. J. Pharmacol. 6, 241–247.

Lüllmann, H., Mohr, K., Pfeffer, J., 1988. Release of *N*-[^{3}H]methylscopolamine from isolated guinea pig atria is controlled by diffusion and rebinding. J. Pharmacol. Exp. Ther. 247, 710–714.

Micheletti, R., Montagna, E., Giachetti, A., 1987. AF-DX 116, a cardioselective muscarinic antagonist. J. Pharmacol. Exp. Ther. 241, 628–634.

Mitchelson, F., 1975. Antimuscarinic action of an alkane-bis-ammonium compound alone and in combination with (+)-benzetimide. Eur. J. Pharmacol. 33, 237–246.

Paton, W.D.M., Rang, H.P., 1965. The uptake of atropine and related drugs by intestinal smooth muscle of the guinea pig in relation to acetylcholine receptors. Proc. R. Soc. London, Ser. B: Biol. Sci. 163, 1–44.

Pedder, E.K., Eveleigh, P., Poyner, D., Hulme, E.C., Birdsall, N.J.M., 1991. Modulation of the structure–binding relationships of antago-

nists for muscarinic acetylcholine receptor subtypes. Br. J. Pharmacol. 103, 1561–1567.

Pitschner, H.F., Schlepper, M., Schulte, B., Volz, C., Palm, D., Wellstein, A., 1989a. Selective antagonists reveal different functions of M cholinoceptor subtypes in humans. Trends Pharmacol. Sci. 10, 92–96 (suppl.).

Pitschner, H.F., Schulte, B., Schlepper, M., Palm, D., Wellstein, A., 1989b. AF-DX 116 discriminates heart from gland M_2-cholinoceptors in man. Life Sci. 45, 493–498.

Roffel, A.F., Elzinga, C.R.S., Meurs, H., Zaagsma, J., 1989. Allosteric interactions of three muscarine antagonists at bovine tracheal smooth muscle and cardiac M_2 receptors. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 172, 61–70.

Schultz, B., Volz-Zang, C., Mutschler, E., Horne, C., Palm, D., Wellstein, A., Pitschner, H.F., 1991. AF-DX 116, a cardioselective muscarinic antagonist in humans: pharmacodynamic and pharmacokinetic properties. Clin. Pharmacol. Ther. 50, 372–378.

Tränkle, C., Mohr, K., 1997. Divergent modes of action among cationic allosteric modulators of muscarinic M_2 receptors. Mol. Pharmacol. 51, 674–682.

Tränkle, C., Andresen, I., Lambrecht, G., Mohr, K., 1998. M_2 receptor binding of the selective antagonist AF-DX 384: possible involvement of the common allosteric site. Mol. Pharmacol. 53, 304–312.

Tucek, S., Proška, J., 1995. Allosteric modulation of muscarinic acetylcholine receptors. Trends Pharmacol. Sci. 16, 205–212.