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Interactions of agonists with an allosteric antagonist at muscarinic acetylcholine M_2 receptors

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Abstract

The interaction of heptane-1,7-bis(dimethyl-3′-phthalimidopropylammonium bromide) (C_7/3′-phth), with several agonists, was investigated at the muscarinic M_2 receptor in guinea-pig left atria. C_7/3′-phth shifted concentration-response curves for the agonists, carbachol, oxotremorine-M and (+)-*cis*-dioxolane, to the right in a parallel fashion. Arunlakshana-Schild regressions of the data yielded slopes significantly different to unity, suggesting non-competitive antagonism. Non-linear regression analysis, using an equation based on allosteric modulation, gave quantitative estimates of co-operativity (α values) and the dissociation constant of C_7/3′-phth (K_B). In all cases, the K_B estimates for C_7/3′-phth were not significantly different. Increasing the carbachol contact time 10-fold did not significantly influence the K_B or the α value obtained with C_7/3′-phth. Changing from Krebs to Tyrode solution did not significantly alter the K_B for C_7/3′-phth, although α values obtained were consistently lower in Tyrode solution, suggesting that the allosteric action may be sensitive to buffer composition. A 4-fold higher degree of negative, heterotropic co-operativity between C_7/3′-phth and agonists than between C_7/3′-phth and competitive antagonists was also found.

Keywords: Muscarinic acetylcholine receptor agonist; Bisquaternary allosteric antagonist

1. Introduction

Competitive antagonists, theoretically, have an unlimited capacity to cause rightward shifts of the concentration-response curve of an agonist. In contrast, allosteric antagonists are characterised by a restricted ability for inhibition. Whilst the latter produce parallel, dextral shifts of the concentration-response curve of an agonist, the extent of the shift reaches a limit at high concentrations of the antagonist (Ariëns et al., 1956; Van den Brink, 1977; Ehlert, 1988). Consequently, an allosteric antagonist produces a curvilinear Arunlakshana-Schild plot (Arunlakshana and Schild, 1959), from which both the dissociation constant and the magnitude of heterotropic co-operativity can be estimated.

Since allosteric antagonism involves a conformational change of the primary, orthosteric, binding site for the agonist, it is possible that different agonists will be affected by such an antagonist to variable degrees. This could occur if the attachment points within the binding site vary in their importance for individual agonists. Allosteric antagonists are known to influence the binding of competitive antagonists to varying extents (Lee and El-Fakahany, 1988, 1991), presumably because the conformational change that the allosteric antagonist induces does not uniformly affect the various attachment points within the binding site for competitive antagonists. This is reflected as variations in the degree of negative, heterotropic co-operativity observed between the allosteric antagonist and different, competitive antagonists. For example, gallamine, a drug known to act allosterically at muscarinic receptors, has been shown to produce a greater degree of negative co-operativity with *N*-methylquinuclidinyl benzilate than with *N*-methylscopolamine (Lee and El-Fakahany, 1988). Some evidence exists that agonists are also influenced to varying extents by gallamine. The antagonist was shown to produce a greater degree of negative co-operativity with carbachol than with acetylcholine and this could not be attributed to any influence of cholinesterases on the responsiveness of acetylcholine (Clark and Mitchelson, 1976).

The bis-quaternary phthalimidopropyl derivative, heptane-1,7-bis(dimethyl-3′-phthalimidopropylammonium bromide) (C_7/3′-phth), is another allosteric muscarinic recep-

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tor antagonist (Lüllmann et al., 1969). $C_7/3'$-phth produces parallel, dextral shifts of the concentration-response curve for carbachol in isolated left atria of the guinea-pig, but the resulting Arunlakshana-Schild plot trends towards a limiting, maximal value at high antagonist concentrations (Lüllmann et al., 1969; Mitchelson, 1975). In experiments with competitive antagonists, $C_7/3'$-phth has been shown to produce variable degrees of negative co-operativity. For example, it produced an about 7-fold lower degree of negative co-operativity in combination with *N*-methylscopolamine than with pirenzepine at the M_2 muscarinic receptor (Christopoulos and Mitchelson, 1994).

The objective of this study was to investigate the allosteric action of this compound with the agonists, acetylcholine, carbachol, oxotremorine-M and (+)-*cis*-dioxolane, to determine whether the allosteric antagonist exhibited different degrees of negative heterotropic co-operativity with the various agonists.

2. Materials and methods

2.1. Muscle bath preparations

Guinea-pigs of either sex were killed by cervical dislocation, their hearts rapidly removed and placed in ice-cold Krebs solution of the following composition (in 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, attached to a tissue hook on the end of an electrode assembly and placed in a 20-ml organ bath containing Krebs solution at 37°C, bubbled with a mixture of 95% O_2 and 5% CO_2. A Grass force-displacement transducer (FT.03 C), 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, 5–15 V).

The tissue was allowed to equilibrate for 20 min under a resting tension of 1 *g* before exposure to an agonist. Except where indicated, a contact time of 40 s with the tissue was employed for each of the agonists. The tissue was washed twice with fresh Krebs solution following each dose of agonist, with 5-min periods allowed between additions of agonist.

At least 3 concentrations of the agonist, with responses obtained in duplicate, were used to construct a reproducible, control concentration-response curve ranging from 20 to 80% of maximal inhibition of atrial contraction. $C_7/3'$-phth was allowed to equilibrate with the tissue for 40 min before a further agonist concentration-response curve was determined.

Some experiments, with acetylcholine as the agonist, were conducted in tissues pretreated with dyflos, 10 μM, for 20 min, with a subsequent 20-min wash period before additions of the agonist.

Experiments were also conducted with an increased contact time for carbachol. The atrial tissue was exposed to the agonist for periods of 10 min throughout the experiment, and the concentration-response curves for the 10-min contact time were compared with those for a 1-min contact time, before and after addition of the antagonist.

Some experiments were conducted in Tyrode solution of the following composition (in mM): NaCl 136.9, KCl 12.1, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 2.0, glucose 5.5 and $CaCl_2$ 1.8, for comparison with results obtained from experiments conducted in Krebs solution.

2.2. Data analysis

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

$$Y = \frac{100 \times [X]}{EC_{50} + [X]}$$

where Y is the response expressed as percentage of inhibition of atrial contractility and X is concentration of agonist. The same procedure was followed for determining EC_{50} values for agonists in the presence of antagonists.

The resulting dose ratios obtained were used to construct an Arunlakshana-Schild plot for the effect of $C_7/3'$-phth against each agonist by fitting a linear regression of log (dose ratio − 1) vs. log [antagonist] through the data points. The slopes obtained were compared to a value of unity, expected for a competitive antagonist. Non-linear regression analysis was also performed on the data, using an equation formulated for an allosteric interaction (Ehlert, 1988):

$$DR - 1 = \frac{(\alpha - 1)}{\left(\frac{\alpha K_B}{B} + 1\right)}$$

where DR represents dose ratio, B the concentration of $C_7/3'$-phth, K_B is the dissociation constant and α is the co-operativity factor.

Pooled dose ratios are expressed as geometric means together with 95% confidence limits; other data are given as means ± S.E.M. Statistical significance was determined using Student's *t*-test. The program, Multcomp, based on a multiple comparison test (Miller, 1966), was employed when comparisons of group means were made. In both cases, values of $P < 0.05$ were considered significant.

2.3. Drugs used

Drugs used were: acetylcholine chloride, carbamylcholine chloride (carbachol), dyflos (Sigma, St. Louis. MO, USA), oxotremorine methiodide (oxotremorine-M), (+)-*cis*-dioxolane (Research Biochemicals International,

Natick, MA, USA) and C_7/3′-phth (heptane -1,7-bis(dimethyl-3′-phthalimidopropyl)ammonium bromide) (Institute of Drug Technology, Boronia, Australia).

3. Results

3.1. Interaction between C_7/3′-phth and muscarinic receptor agonists

Concentration-response curves for the negative inotropic effect of the various muscarinic receptor agonists were shifted to the right in a parallel fashion by a range of concentrations of C_7/3′-phth. Arunlakshana-Schild plots of the data revealed a limited ability of C_7/3′-phth to produce parallel, dextral displacements of the agonist concentration-response curves, as indicated in Fig. 1, for acetylcholine. Linear regressions for each agonist, plotted through all the data points, gave slopes significantly < 1 (Table 1).

Another fit of the data (Figs. 1 and 2) was obtained with a non-linear regression for C_7/3′-phth derived for an allosteric model (see Section 2). Table 2 compares the goodness-of-fit (r^2) parameters of the two regressions for the effect of C_7/3′-phth with each agonist. Estimates of the dissociation constant for C_7/3′-phth and the co-operativity factor (α) for the interaction between C_7/3′-phth and each of the agonists were also obtained (Table 3). There was no significant difference ($P > 0.05$) in the estimates of the dissociation constant obtained for C_7/3′-phth with any of the agonists employed. However, the magnitude of the negative co-operativity for the interaction between C_7/3′-phth and acetylcholine was significantly different ($P < 0.05$) from α values obtained for C_7/3′-phth with the other three agonists, carbachol, oxotremorine-M and (+)-*cis*-dioxolane.

Some experiments were performed in the presence of dyflos to minimise any possible anticholinesterase activity of C_7/3′-phth, and an Arunlakshana-Schild plot was constructed with four concentrations of the antagonist (Fig. 3).

Table 1
Arunlakshana-Schild plot slope parameters for interaction between C_7/3′-phth and various muscarinic receptor agonists in guinea-pig atria

Agonist	Krebs solution		Tyrode solution	
	Slope ± S.E.M. [a]	n [b]	Slope ± S.E.M. [a]	n [b]
Acetylcholine	0.68 ± 0.06 [d]	8	0.53 ± 0.11 [c]	5
Acetylcholine + dyflos	0.85 ± 0.04 [c]	4	–	–
Carbachol	0.82 ± 0.02 [d]	5	0.75 ± 0.05 [d]	5
Oxotremorine-M	0.88 ± 0.04 [c]	5	0.75 ± 0.04 [d]	5
(+)-*cis*-Dioxolane	0.81 ± 0.04 [d]	5	0.73 ± 0.05 [c]	4

[a] Slope ± S.E.M. of Arunlakshana-Schild plots as determined by computer analysis.
[b] Number of data points (i.e. concentrations of C_7/3′-phth).
[c] Significantly different from 1 ($P < 0.05$).
[d] Significantly different from 1 ($P < 0.01$).

Table 2
Goodness-of-fit (r^2) parameters for regression analyses in different buffers for C_7/3′-phth with various muscarinic receptor agonists

Agonist	Krebs solution		Tyrode solution	
	Linear	Non-linear	Linear	Non-linear
Oxotremorine-M	0.9938	0.9987	0.9909	0.9968
Acetylcholine + dyflos	0.9964	0.9979	–	–
Carbachol	0.9974	0.9993	0.9850	0.9982
(+)-*cis*-Dioxolane	0.9940	0.9932	0.9769	0.9993
Acetylcholine	0.9490	0.9988	0.8782	0.9901

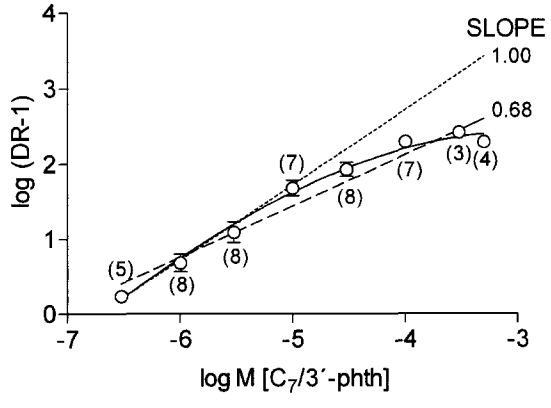

Fig. 1. Arunlakshana-Schild plot for the effect of C_7/3′-phth using acetylcholine as agonist. Each point represents the geometric mean dose ratio; the number of experiments is indicated in parentheses. The linear regression through the data points (- - -) has a slope of 0.68 as indicated. A non-linear regression through the data points (——), based on an allosteric interaction (see Section 2), with a K_B value of 189.6 nM and an α value of 268.2 is also shown. A line with the same K_B value and a slope of unity (. . .) is shown for comparison. Where S.E. bars are not shown, they lie within the dimensions of the symbol.

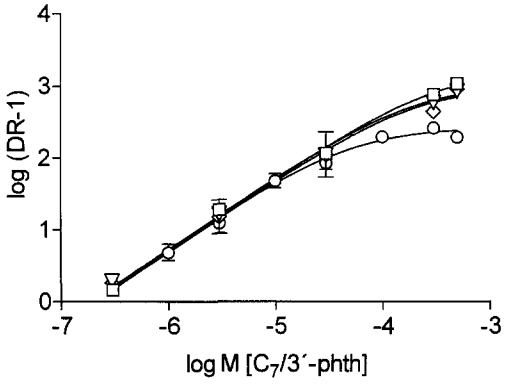

Fig. 2. Arunlakshana-Schild plot for C_7/3′-phth with various agonists; acetylcholine (○), carbachol (▽), (+)-*cis*-dioxolane (◇) and oxotremorine-M (◇). Each point represents the mean result obtained from 3–8 determinations except for C_7/3′-phth (500 μM) with (+)-*cis*-dioxolane as agonist where only one determination was made. Non-linear regressions based on an allosteric interaction are plotted for each data set. Other details as in Fig. 1.

Table 3
Estimates (mean ± S.E.M.) of co-operativity factors (α) and K_B values for C_7/3′-phth with various muscarinic receptor agonists in Krebs solution

Agonist	α	K_B (nM)	n [a]
Oxotremorine-M	1723.1 ± 457.4	194.4 ± 22.0	8
Acetylcholine + dyflos	1679.9 ± 1061.1	172.0 ± 42.5	4
Carbachol	1063.4 ± 265.0	186.2 ± 23.4	5
(+)-*cis*-Dioxolane	1014.7 ± 299.1	205.3 ± 46.5	5
Acetylcholine	268.2 ± 27.2 [b]	189.6 ± 18.2	5

[a] Number of data points (i.e., concentrations of C_7/3′-phth).
[b] Significantly different from other α values ($P < 0.05$).

The co-operativity factor (α) between C_7/3′-phth and acetylcholine in the presence of dyflos was significantly higher than in the absence of the anticholinesterase ($P < 0.05$), due to the greater dose ratios achieved with the two higher concentrations of the antagonist used in the presence of dyflos. However, there was no significant difference between the K_B values ($P > 0.05$) (Table 3, Fig. 3). Furthermore, the magnitude of negative co-operativity between C_7/3′-phth and acetylcholine, in the presence of dyflos, was not significantly different ($P > 0.05$) from that obtained with the other agonists (Table 3).

3.2. Alteration of agonist contact time

Allosteric antagonists may slow the rate of equilibration of an agonist with its binding site and, consequently, a longer time may be required for the full response to be attained. Using carbachol as the agonist, experiments were conducted where the contact time was varied 10-fold. Measurement of the response to carbachol at 1 and 10 min did not significantly influence the K_B or α values for C_7/3′-phth ($P > 0.05$). The 1- and 10-min contact times gave K_B values of 141.5 ± 17.7 and 166.1 ± 36.5 nM, respectively, and α values of 1177.0 ± 262.2, 5 (mean ± S.E.M., number of data points) and 948.6 ± 363.3, 5, respectively. Furthermore, none of these values differed significantly ($P > 0.05$) from the corresponding estimate using a 40-s contact time for the agonist (see Table 3).

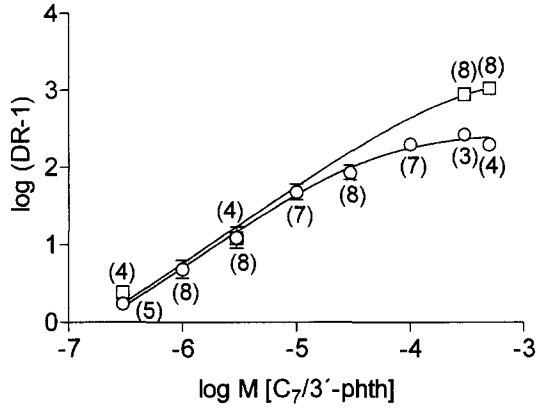

Fig. 3. Arunlakshana-Schild plots for C_7/3′-phth with acetylcholine as agonist, in the absence (○) and presence (□) of dyflos. Number of determinations are noted in parentheses alongside each of the data points. Other details as in Fig. 1.

3.3. Change of buffer composition

Comparable experiments conducted in Tyrode solution suggested that the negative co-operativity of C_7/3′-phth was influenced by a change in buffer composition. In these experiments, the dissociation constant for C_7/3′-phth did not vary significantly ($P > 0.05$) from that in Krebs solution, using any agonist. However, the negative co-operativity between C_7/3′-phth and acetylcholine was significantly lower in Tyrode solution ($P < 0.05$) (Table 4). A similar trend for C_7/3′-phth with the other three agonists in Tyrode solution was also observed. The dose ratios obtained with the highest concentration of C_7/3′-phth (500 μM) in Tyrode solution differed significantly ($P < 0.05$) from the corresponding value in Krebs solution (with the exception of (+)-*cis*-dioxolane) (Table 4) but the estimated α values for C_7/3′-phth with the various agonists in Tyrode solution did not ($P > 0 < .05$).

3.4. Comparative analyses with antagonists

The α values obtained in the present study were compared with values obtained for C_7/3′-phth with antagonists

Table 4
Comparison of estimated co-operativity factors (α) and measured maximal dose ratios (DR) for C_7/3′-phth with various muscarinic receptor agonists in Krebs and Tyrode solution

	Krebs solution		Tyrode solution	
Agonist	α [a]	DR [b] C_7/3′-phth (500 μM)	α [a]	DR [b] C_7/3′-phth (500 μM)
Oxotremorine-M	1723 ± 457 (5)	1101.8 (1209.8–1003.0; 3)	719 ± 242 (5)	597.0 [d] (693.9–515.4; 4)
Carbachol	1063 ± 265 (5)	859.7 (935.5–792.1; 8)	596 ± 121 (5)	496.1 [d] (584.0–423.1; 3)
(+)-*cis*-Dioxolane	1015 ± 299 (5)	923.0 (1)	371 ± 120 (5)	357.1 (470.8–272.4; 3)
Acetylcholine	268 ± 27 (8)	197.4 (213.0–184.8; 4)	126 ± 34 [c] (5)	86.3 [d] (15.9–13.4; 3)

[a] Mean ± S.E.M. (number of data points); each data point represents a different concentration of C_7/3′-phth used.
[b] Geometric mean (95% confidence limits; number of determinations).
[c] Significantly different ($P < 0.05$) from α value using acetylcholine in Krebs solution.
[d] Significantly different ($P < 0.05$) from corresponding dose ratio in Krebs solution.

Table 5
Comparison of co-operativity factors (α) for $C_7/3'$-phth with various muscarinic receptor agonists vs. various muscarinic receptor antagonists

Agonist	α	Antagonist	α [a]
Oxotremorine-M	1723.1 ± 457.4	Pirenzepine	58.2 ± 12.8
Acetylcholine + dyflos	1679.9 ± 1061.1	Homatropine	39.7 ± 5.0
Carbachol	1063.4 ± 265.0	Atropine	33.1 ± 6.3
(+)-*cis*-Dioxolane	1014.7 ± 299.1	Dexetimide	25.9 ± 4.0
Acetylcholine	268.2 ± 27.2	*N*-Methyl-scopolamine	8.8 ± 1.6

[a] Data obtained from Christopoulos and Mitchelson (1994).

from a previous study (Christopoulos and Mitchelson, 1994) (Table 5). It was evident that the degree of negative co-operativity between $C_7/3'$-phth and the agonists is much greater than those between $C_7/3'$-phth and the competitive antagonists (Table 5).

4. Discussion

$C_7/3'$-phth was found to produce Arunlakshana-Schild plots with slopes significantly less than unity when used with each of the agonists in Krebs solution, suggesting that the nature of the antagonism was not competitive. This was attributed to lower than expected dose ratios for the higher concentrations of $C_7/3'$-phth employed, indicating that the inhibition of the responses to muscarinic receptor agonists was reaching a limiting value. The effect on slope was most marked with acetylcholine and least with oxotremorine-M as the agonist.

Since acetylcholine, in contrast to the other agonists studied, is susceptible to hydrolysis by cholinesterases, it was possible that $C_7/3'$-phth was exerting some inhibition of cholinesterase activity which counteracted its ability to inhibit muscarinic receptors. This was supported by experiments carried out in the presence of dyflos. The dose ratios produced by the higher concentrations of $C_7/3'$-phth with acetylcholine were greatly increased in the presence of dyflos, so that limiting dose ratios for $C_7/3'$-phth obtained with acetylcholine as agonist were similar to those with oxotremorine-M.

Ehlert (1988) has described a theoretical basis for an antagonist acting as a negative allosteric modulator and has shown that the corresponding theoretical Arunlakshana-Schild plot, of log (dose ratio − 1) vs. log [antagonist], will be curvilinear and reach a limiting value at high concentrations of antagonist. The intercept of the plot with the x-axis provides a measure of the dissociation constant, as in an Arunlakshana-Schild plot for a competitive antagonist, and the limiting value to which the plot trends, at higher concentrations of the antagonist, is a measure of the degree of negative heterotropic co-operativity between the allosteric ligand acting at a secondary site and the agonist binding at the orthosteric (primary) site. Fitting such a regression to the data obtained with $C_7/3'$-phth, using each of the agonists, produced a measure of the dissociation constant (K_B value) for $C_7/3'$-phth and a co-operativity factor (α value); a measure of the interaction between the allosteric and orthosteric ligands. Statistical comparison showed no difference in the estimates of the K_B value obtained for $C_7/3'$-phth nor the α values, with any of the agonists, providing the data for acetylcholine in the presence of dyflos was used.

Allosteric antagonists may slow the rate of equilibration of drugs at the orthosteric site (Stockton et al., 1983; Jepsen et al., 1989) and, therefore, an agonist may require a longer period to exert its full effect in the presence of the allosteric antagonist. However, in the present study, it was found that increasing the contact time up to 10 min for carbachol with the atrial tissue, did not influence the ability of $C_7/3'$-phth to shift the concentration-response curves to the right. Therefore, a 40- or 60-s contact time of carbachol with the tissue appeared adequate to replicate concentration-response curves for the agonist in the presence of $C_7/3'$-phth. Although $C_7/3'$-phth has been found to slow the association and dissociation of competitive antagonists (Jepsen et al., 1989; Christopoulos and Mitchelson, 1994, 1995), it is possible that this effect is less marked in the case of agonists, and the present results are in accordance with this suggestion.

Krebs solution was used for the majority of the experiments with $C_7/3'$-phth and the agonists. In some experiments, Tyrode solution was employed in place of Krebs solution, to investigate the effect of varying the ionic composition of the buffer. It was found that with Tyrode solution, as for Krebs solution, the maximum dose ratio for $C_7/3'$-phth with acetylcholine was significantly smaller than that with oxotremorine-M. A difference between the buffers was that dose ratios obtained for the maximum concentration of $C_7/3'$-phth (500 μM) with the agonists, oxotremorine-M, carbachol and acetylcholine, were significantly smaller in Tyrode solution. Subsequent non-linear regression analysis showed that changing from Krebs to Tyrode solution did not significantly alter the estimates of the dissociation constant for $C_7/3'$-phth using any of the agonists. However, the α value obtained for $C_7/3'$-phth with acetylcholine was significantly smaller in Tyrode solution. A similar trend for a smaller α value of $C_7/3'$-phth in Tyrode solution was observed with each of the other agonists, although the differences were not statistically significant. It was considered that the difference between the two buffers may have been due to the 4.5-fold difference in K^+ concentration, as some allosteric muscarinic receptor antagonists are known to produce blockade of ion channels (Freeman and Dawson, 1991; Lee and El-Fakahany, 1991; Dunn et al., 1996). However, lowering the K^+ concentration of the Tyrode solution to 2.7 mM in two experiments did not alter the K_B or α value obtained

for $C_7/3'$-phth using carbachol as the agonist (data not shown). Recently, Burgmer et al. (1996) have suggested that magnesium may compete for the allosteric site and reduce the degree of slowing of [^{3}H]*N*-methylscopolamine dissociation induced by an allosteric antagonist. However, this did not appear to be the reason for the differences observed since the Tyrode solution contained only an about 15% lower magnesium concentration and, in any case, lower α values were obtained for the antagonist in Tyrode solution than in Krebs solution.

Previous studies investigating the interaction of $C_7/3'$-phth with competitive antagonists yielded lower α values (Mitchelson, 1975; Christopoulos and Mitchelson, 1994) in comparison to the α values obtained for $C_7/3'$-phth with the agonists in the present study. In the case of antagonists, there was an about 7-fold difference in estimates of α values for the interaction between $C_7/3'$-phth and *N*-methylscopolamine than between the allosteric antagonist and pirenzepine (8.8 vs. 58.4). These findings suggested that $C_7/3'$-phth induced a much greater conformational change at the binding points for pirenzepine than for *N*-methylscopolamine. In comparison, there was no more than a 1.7-fold difference in the α values for $C_7/3'$-phth with the various agonists. Thus, the conformational changes at the attachment points of agonists within the orthosteric site were similar, although small variations must exist, given the significant differences in dose ratios obtained with the highest concentration of $C_7/3'$-phth and the various agonists in Tyrode solution.

In conclusion, $C_7/3'$-phth produced non-linear Arunlakshana-Schild plots with all the agonists investigated. No significant differences were obtained for the degree of negative, heterotropic co-operativity between $C_7/3'$-phth and any of the agonists, although these values were greater than those obtained in previous studies for the interaction of $C_7/3'$-phth and competitive antagonists.

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