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HUMAN IDIOPATHIC AND NEUROGENIC OVERACTIVE BLADDER AND THE ROLE OF M2 MUSCARINIC RECEPTORS IN CONTRACTION

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ABSTRACT

Objectives : The study examines whether M_2 receptors contribute to the direct contraction of the detrusor in the human neurogenic and idiopathic overactive bladder.

Methods: Control detrusor muscle was obtained from patients undergoing cystectomy for bladder cancer, whilst overactive detrusor muscle was obtained from patients undergoing clam cystoplasty for idiopathic or neurogenic detrusor overactivity. The affinities of a range of subtype selective antagonists (DAMP, darifencain, methoctramine R0-320-6206 and pirenzepine) were obtained in tissue bath experiments using carbachol as the agonist. These affinity values were then compared with the known affinities for these antagonists at the muscarinic receptor subtypes.

Results: An increased sensitivity to carbachol was observed in both the neurogenic and idiopathic overactive detrusor compared to the control human detrusor. The M_2 selective antagonists (methoctramine, R0-320-6206) and M_1 selective antagonist (pirenzepine) had low affinities, whilst the M_3 selective antagonists (4-DAMP and darifenacin) had high affinities for the human detrusor muscarinic receptor in all 3 groups of tissues. The affinities (pK_B values) for the five antagonists were consistent with antagonisms at the M_3 receptor in all three groups and Schild plot analysis indicated an action at this single receptor subtype.

Conclusion: Muscarinic receptor mediated function is enhanced in the idiopathic and neurogenic overactive compared to control detrusor. The direct contractile response to carbachol is mediated by the M_3 receptor in both the human normal and overactive bladder indicating no change in receptor subtype contribution to contraction in the disease state.

Key words : Overactive bladder, neurogenic, idiopathic, muscarinic receptors.

INTRODUCTION

Muscarinic antagonists are the mainstay of treatment for the overactive detrusor, yet little is known about the mechanisms involved in initiating detrusor contraction following muscarinic receptor stimulation. It has been suggested that ATP plays an enhanced role in mediating contraction in the overactive detrusor, but acetylcholine acting via muscarinic receptors remains the predominant mechanism of contraction in both the normal and overactive bladder in man [1].

Human bladder tissue contains a heterogeneous population of M_2 and M_3 receptors with a ratio of 3:1 indicating the predominance of the M_2 subtype [2]. In human tissue M_3 but not M_2 antagonists have a high affinity for the receptor mediating contraction indicating in vitro functional responses to muscarinic receptor stimulation is mediated via the minor population of M_3 muscarinic receptors [3-5]. Similar findings have been reported in number of other species (for review see [6]).

Cystometric studies in M_2 and M_3 knock out mice also indicate the M_3 receptor as the dominant subtype *in vivo* [7]. Thus, the functional role of the large population of M_2 receptors in the bladder is not fully understood. In rat models of neurological damage a change in muscarinic receptor function has been observed, with bladders from dennervated and spinal cord injured rats exhibiting an increased sensitivity to muscarinic agonist stimulation, an enhanced M_2 receptor expression and a shift in the affinity of antagonists to indicate a role of M_2 receptors as well as M_3 in direct smooth muscle contraction [8, 9]. Research from the same laboratory has also implicated M_2 receptor involvement in direct contraction of human neurogenic bladder dysfunction [10].

Muscarinic receptor mediated responses play a key role in bladder contractility and alterations in muscarinic receptor function therefore may be important to the understanding of the disease state. The aim of the present study was to investigate the role of M_2 receptors in the contraction of the human neurogenic overactive bladder and of the idiopathic overactive bladder. This was achieved by comparing the *in vitro* characteristics of contraction to carbachol in human overactive detrusor compared to control human detrusor.

METHODS

Approval for this study was obtained from the South Sheffield Ethics Committee. All tissues were obtained as remnants from surgery and patient consent had been given. Tissues representing control detrusor were obtained from patients undergoing cystectomy procedures for bladder cancer in which the whole bladder was removed. A section of bladder dome tissue from an area free from cancer was dissected for the study. Tissues from a total of 15 control patients were studied, 10 male (mean age = 62 years, range 39 to 72 years) and 5 female (mean age = 51 years, range 31 to 70 years). Samples of neurogenic overactive bladder tissue were obtained from spinal injury patients undergoing surgical procedures such as cystectomy, urinary diversion procedures or clam cystoplasy to alleviate unwanted bladder symptoms. In addition to the spinal injury patients, one patient with overactivity due to cerebral palsy was included in the study. A total of 10 neurogenic patients were studied, 8 male (mean age = 32 years, range 13 to 49 years) and 2 female (31 and 61 years). Patients with an idiopathic condition had no known clinical cause for their overactive detrusor. These patients had previously tried drug therapy such as muscarinic antagonist to control their symptoms, but treatment had failed due to lack of efficacy or side effects. The use of anticholinergic therapy in this patient group had been terminated before proceeding to the operation. Patients in the idiopathic group were undergoing clam cystoplasty, and a total of 10 patients were studied; 8 female (mean age = 55 years, range 35 – 68 years) and 2 male (33 and 57 years).

Strips of tissue (7mm x 5mm) were dissected and the serosa, suburothelium and urothelium removed. The tissues were mounted in 30ml organ baths containing Krebs bicarbonate solution which was maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. The tissues were subjected to a resting tension of 1g and allowed to equilibrate for 60 minutes and during this time tissues were washed every 15 minutes. Isometric tension generated by the tissues was measured using UFI force transducers connected to a PC via a Cambridge electronic design (CED) interface using CHART software.

Following the equilibration period, cumulative concentration-response curves to carbachol were obtained. Tissues were then washed for 45 minutes until the tension had returned to baseline values and were then incubated for 30 minutes with the appropriate concentration of antagonist or vehicle (time control). Four concentration-response curves to carbachol were obtained on each tissue, washing between each; one control curve and three curves in the presence of increasing concentrations of antagonist. The subtype selective muscarinic antagonists included in the study were the M_3 selective 4-DAMP (3-30nM) and darifenacin (3-100nM); the M_2 selective antagonists methoctramine (1-100 μM) and RO-320-6206 (30-300nM, 1-10μM) and the M_1 selective antagonist pirenzepine (3-30μM). Control experiments, where no antagonist was added, were performed in each tissue category to correct for any time-dependant changes in tissue sensitivity and maxima during the course of the experiments. These values were similar in control, neurogenic and idiopathic groups (eg. For pEC50 values the shift was 0.4. 0.4 and 0.3 respectively between first and second curves). All samples were weighed following experimentation.

Statistical analysis

Contractile responses to carbachol in the different experimental groups were compared relative to the tissue weight. Increases in developed tension to carbachol in the absence and presence of antagonist were plotted as a percentage of the maximum increase for each concentration-response curve. Individual EC_{50} values (molar concentration producing half-maximal response) were determined by non-linear regression fitting (variable slope) to the concentration response curves by GraphPad Prism software. Data from individual curves were used to derive mean –log EC_{50} (pEC_{50}) values with s.e.mean. Dissociation constants (pK_B) for antagonists were determined from the equation:

$$pK_B = \log (CR-1)-\log[B]$$

where CR is the concentration ratio (ratio of the EC_{50} values) in the absence and presence of the antagonist obtained with a concentration [B] of antagonist. Schild analysis was also performed and the gradient used to assess the competitive nature of the antagonism [11]. Apparent pK_B values are given in situations where only one concentration of antagonist was tested.

Differences in pEC_{50} and maximum responses between the detrusor groups were determined by ANOVA followed by Bonferroni multiple comparison post hoc test. Differences between mean maximum responses for control curves and those in the presence of the highest concentration of antagonist were compared using the paired Students t-test.

Drugs

Carbachol (carbamylcholine chloride), 4-DAMP (4-diphenylacetoxy-N-methyl-piperadine methiodide), pirenzepine and methoctramine, were obtained from Sigma-Aldrich (Poole, UK). RO 320-6206 ((R)-4-piperidine-1-carboxylic acid amide) was synthesized as described by [12-14] .Darifenacin was a gift from Pfizer (Sandwich, UK). All solutions were freshly prepared in distilled water and dilutions made in Krebs bicarbonate solution.

RESULTS

Cumulative application of carbachol evoked concentration-dependant contractions of the detrusor smooth muscle strips in all three groups of tissues. In the neurogenic bladder the mean pEC50 (6.57 ± 0.08, n = 33) was significantly greater ($p<0.001$) compared to the normal bladder (5.94 ± 0.07, n = 37) indicating an increased sensitivity of the neurogenic overactive detrusor to carbachol (Figure 1). An increase in tissue sensitivity to muscarinic stimulation was also observed in the idiopathic overactive bladder where the pEC50 (6.18 ± 0.05, n = 35) was significantly greater ($p<0.05$) than the control human detrusor (Figure 1). Mean maximal responses of the bladder tissue expressed as grams comparative to the tissue weight were not significantly different between control detrusor and either the neurogenic or idiopathic detrusor samples (maximal response = 0.22 ± 0.04 g/mg for normal bladder vs 0.24 ± 0.03 g/mg in the neurogenic and 0.24 + 0.04g/mg in the idiopathic overactive bladder).

Effects of muscarinic antagonists

In the control human detrusor, the M_1 selective antagonist pirenzepine, the M_2 selective antagonist methoctramine and the M_3 antagonists 4-DAMP and darifenacin all acted as competitive antagonists of carbachol induced contractile responses. All four antagonists produced parallel rightward shifts of the concentration-response curves to carbachol without altering maximum responses and produced Schild plots with slopes of unity (Figure 2).

4-DAMP and darifenacin yielded relatively high affinity estimates (9.8 and 8.0 respectively). However, higher concentrations of methoctramine and pirenzepine were required to antagonise the responses to carbachol and these antagonists had relatively low affinities (pK_B 6.0 and 6.7 respectively). RO-320-6206 at concentrations below 1μM was unable to influence contractile responses to carbachol, but a high concentration (10μM) evoked a shift of the concentration-response curve to carbachol generating an apparent pK_B value of 5.8 that is consistent with the antagonism of M_3 receptors.

The same range of antagonists was used to characterise the contractile muscarinic receptor(s) in the neurogenic and idiopathic overactive detrusor. In neurogenic and

idiopathic overactive detrusor, 4-DAMP and darifenacin competitively antagonised carbachol induced contractile responses. The rightward shift in the concentration-response curves to carbachol by 4-DAMP and darifenacin yielded high affinity estimates consistent with the values derived in the control human detrusor (table 1). Methoctramine in the overactive bladder was required at higher concentrations to antagonise carbachol induced contractile responses and from the rightward shifts of the concentration-response curves generated relatively low affinity estimates of 5.7 in the neurogenic detrusor and 6.1 in the idiopathic overactive detrusor. Schild plots for the antagonism of responses by 4-DAMP, darifenacin and methoctramine had slopes not significantly different from unity, indicating that only one receptor subtype was involved in mediating contraction in the overactive bladder (figure 3 and 4). The actions of pirenzepine were also assessed at 3 concentrations (3-30μM) in 2 samples of the neurogenic overactive bladder and 2 samples of the idiopathic overactive bladder. From the available data apparent pK_B values of 6.3 and 6.7 was calculated in the neurogenic and idiopathic overactive bladder respectively (Table 1). The antagonist R0-320-6004 at nanomolar concentrations was unable to inhibit carbachol contraction of the detrusor strips. A high concentration of R0-320-6206 (10μM) evoked a shift of the carbachol contraction response curve and produced a low apparent pK_B values of 5.4 in the neurogenic and 6.0 in the idiopathic overactive bladder.

DISCUSSION

Based on a suggested enhanced M_2 function in animal models of bladder dysfunction [8, 9], the aim of the present study was to assess the role of M_2 receptors in direct contraction in human bladder disease (neurogenic and idiopathic overactive detrusor). Carbachol evoked a concentration-dependant contraction with a similar maximal contraction in all three groups of tissues when assessed relative to the tissue weight. However, in both the neurogenic and idiopathic overactive detrusor there was an increase in sensitivity to the muscarinic agonist which highlights a change in receptor-mediated mechanisms in both disorders of overactivity compared to the normal detrusor. An increased sensitivity to muscarinic agonists has been reported previously in the human neurogenic overactive bladder [15-17] and in rat models of neurogenic overactivity [8]. In the rat models of neurogenic damage (denervated and spinal cord injured) a shift in the affinity of muscarinic antagonists towards M_2 mediated contraction was observed in addition to a muscarinic super sensitivity [8, 9].

In studies of gene knock-out (KO) mice, contraction of the bladder was still possible, albeit minor, in M_3 KO animals compared to wild type mice and in M_2 KO mice, a two-fold decrease in tissue sensitivity to carbachol was observed indicating a possible M_2 mediated direct contraction [18-20]. These observations support the idea of the M_2 receptor coupling to a G-protein pathway linked to direct contraction and this hypothesis was investigated in the present study.

The M_3 antagonists 4-DAMP and darifenacin, antagonised muscarinic contraction with high affinity in the control, neurogenic and idiopathic overactive detrusor; the derived affinities being in agreement with published affinities for the antagonism at M_3 receptors [2, 3, 21-26]. The M_2 antagonist methoctramine antagonised detrusor responses with low affinity, well below that normally observed at M_2 receptors [2, 3, 22-26]. The Schild plots for all three antagonists had slopes not significantly different from unity reflecting an antagonism at one receptor subtype. RO-320-6206, another M_2 receptor selective antagonist, did not influence responses when used at concentrations that selectively inhibit the M_2 receptor subtype and only antagonised responses at a higher concentration (10μM) with an apparent affinity that is consistent with an action at M_3 receptors [13]**.** Pirenzepine (M_1 selective) also antagonised

responses with a relatively low affinity in all the tissues indicates that the involvement of M_1 receptors in contraction of the human overactive bladder can be excluded.

In the control human detrusor, the derived affinity constants for all 5 antagonists were consistent with values for the M3 subtype appearing in the literature. These data suggest that in the human control detrusor the *in vitro* direct contractile responses to muscarinic stimulation are mediated via the M_3 receptor subtype. This result supports previous studies in the human detrusor [3, 4]. In strips of detrusor muscle from neurogenic overactive or idiopathic overactive detrusor patients, there was no evidence of a shift in affinity from M_3 to M_2 for any of the muscarinic antagonist. Furthermore maximum responses to carbachol were not depressed by any of the antagonists and where it was possible to perform Schild analysis, the plots had slopes of unity again consistent with the antagonism of only one receptor population. These results suggest that in both the human neurogenic overactive and idiopathic overactive detrusor the mechanisms of muscarinic contraction remains mediated by the M_3 subtype as found in the human control tissue.

Reports of the role of M_2 receptors in animals models of detrusor overactivity are conflicting. One group has suggested that M_2 receptors contribute to direct detrusor contraction in the denervated, non-voiding spinal injured rat and the obstructed rat [8, 9, 27], whilst others could find no evidence of a change in receptor function in the obstructed rat [28]. In the present study patients with neurogenic overactivity due to spinal injury were able to void and in these patients detrusor contraction remained mediated by the M_3 receptor.

There have been very few studies investigating muscarinic receptor subtype function in the human detrusor, but one study has examined the effects of a single concentration of an M_3 antagonist (darifenacin or p-F-HHSiD) on the contractile responses of tissues from patients with neurogenic overactive bladder (Pontari et al., 2004). The potency of the M_3 antagonist varied between patients and the study concluded that M_2 receptors did play a role in mediating contraction of the neurogenic overactive detrusor.

The present study could find no evidence to support this conclusion. Indeed, all the data, which included a range of concentrations of darifenacin, 4-DAMP and

methoctramine on samples from each patient, confirmed that responses were mediated via M_3 receptors. Furthermore the examination of several concentrations allowed accurate measurements of drug affinity and also the construction of Schild plots which identified only one receptor mediating contraction (M_3). In this comprehensive study identical results were obtained with tissues from control, neurogenic and idiopathic overactive detrusor tissue. Thus, M_2 receptors do not appear to contribute to direct contraction of the human detrusor in normal or disease states.

In conclusion, these data show that carbachol induced contraction of the human detrusor muscle is enhanced in both the neurogenic and idiopathic overactive compared to normal bladder. The direct contractile response to carbachol is mediated by the M_3 receptor in both the human control and overactive detrusor and no evidence could be found to indicate any change in the muscarinic receptor subtype mediating contraction in the human overactive detrusor.

Acknowledgements

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DRUG	TISSUE CATEGORY	AFFINITY (pk_B)	SCHILD SLOPE	n
4-DAMP	Normal	9.83 ± 0.11	0.98 ± 0.01	12
	Neurogenic OA	9.84 ± 0.06	0.88 ± 0.05	15
	Idiopathic OA	9.92 ± 0.11	0.75 ± 0.11	17
Darifenacin	Normal	8.00 ± 0.08	1.06 ± 0.12	13
	Neurogenic OA	8.53 ± 0.08	0.84 ± 0.04	14
	Idiopathic OA	8.51 ± 0.10	0.86 ± 0.13	13
Methoctramine	Normal	6.04 ± 0.12	1.13 ± 0.23	15
	Neurogenic OA	5.69 ± 0.05	0.85 ± 0.05	15
	Idiopathic OA	6.12 ± 0.10	0.88 ±0.24	20
Pirenzepine	Normal	6.65 ± 0.12	1.06 ± 0.12	18
	Neurogenic OA	6.31 ± 0.14	na	6
	Idiopathic OA	6.67 ± 0.11	na	6

Table 1: Antagonist data for human control, neurogenic and idiopathic overactive detrusor muscle. Affinity estimates (apparent pK_B values) and Schild slopes are expressed as mean ± s.em. n is the number of experiments. (na = not applicable, where only one concentration of antagonist was examined).

FIGURE LEGENDS

Figure i: Concentration-response curves for carbachol in human control detrusor, the neurogenic overactive detrusor and the idiopathic overactive detrusor. Responses are expressed as a percentage of the maximum response and presented as mean ± s.e.m.

Figure ii: The antagonism of carbachol concentration-response curves by [A] 4-DAMP and [B] methoctramine in **CONTROL HUMAN DETRUSOR**. Responses are expressed as the mean ± s.e.m. [C] Schild plots for the antagonism by 4-DAMP and methoctramine.

Figure iii: The antagonism of carbachol concentration-response curves by [A] 4-DAMP and [B] methoctramine in **NEUROGENIC HUMAN DETRUSOR**. Responses are expressed as the mean ± s.e.m. [C] Schild plots for the antagonism by 4-DAMP and methoctramine.

Figure iv: The antagonism of carbachol concentration-response curves by [A] 4-DAMP and [B] methoctramine in **IDIOPATHIC HUMAN DETRUSOR**. Responses are expressed as the mean ± s.e.m. [C] Schild plots for the antagonism by 4-DAMP and methoctramine.

Figure i

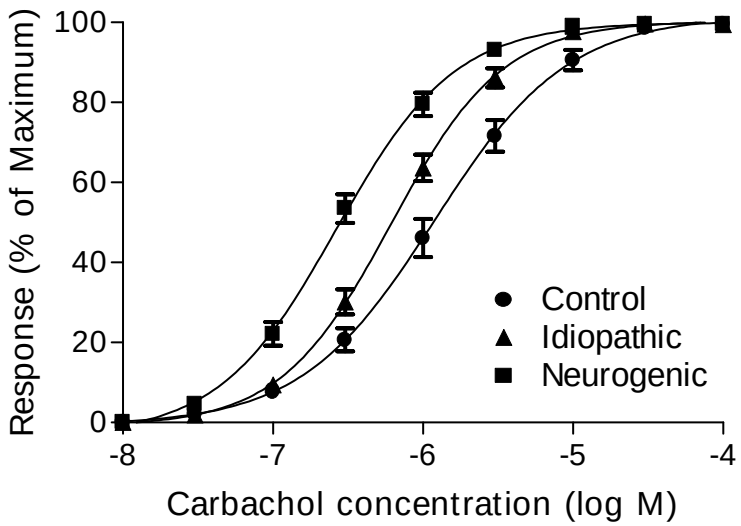

Response (% of Maximum)
100
80
60
40
20
0
-8
-7
-6
-5
-4
Carbachol concentration (log M)
Control
Idiopathic
Neurogenic

Figure ii

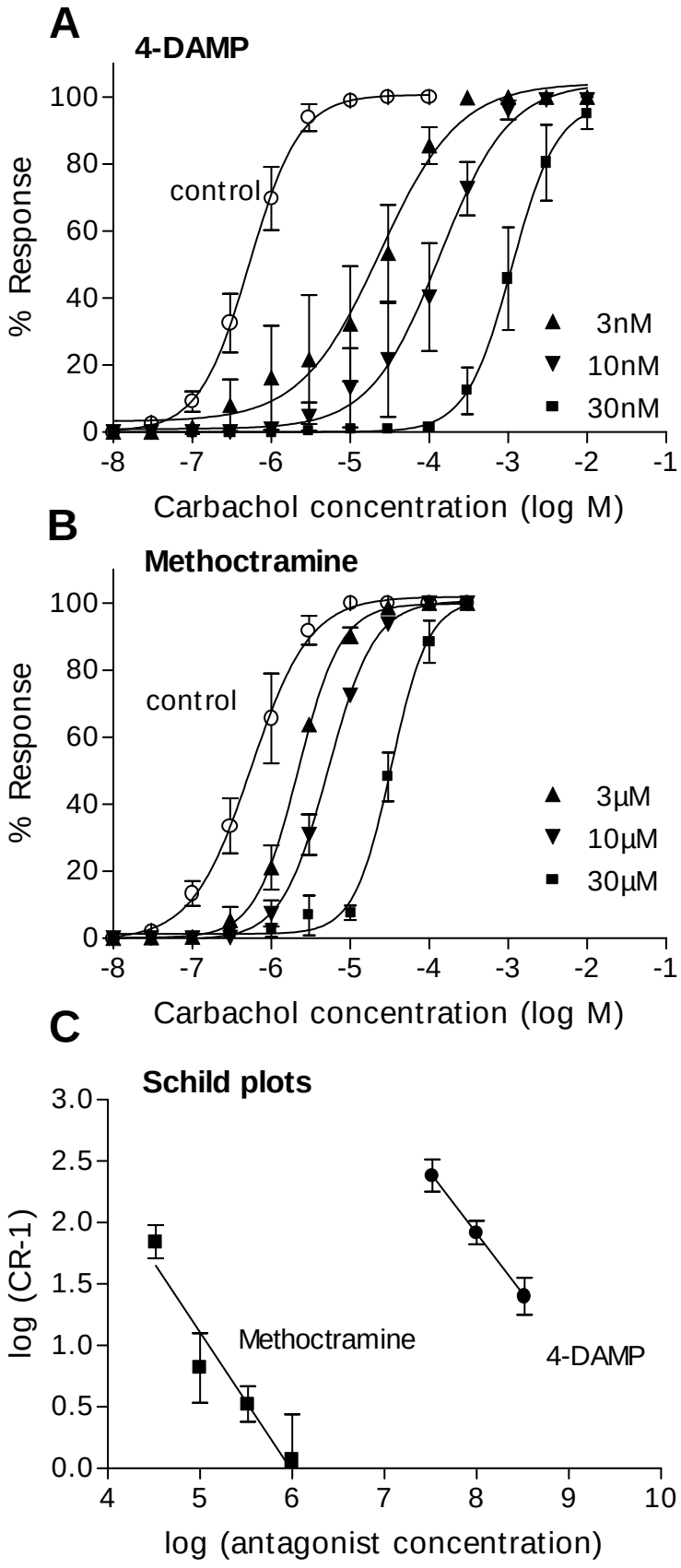

A
4-DAMP
% Response
control
3nM
10nM
30nM
Carbachol concentration (log M)
B
Methoctramine
% Response
control
3µM
10µM
30µM
Carbachol concentration (log M)
C
Schild plots
log (CR-1)
Methoctramine
4-DAMP
log (antagonist concentration)

Figure iii

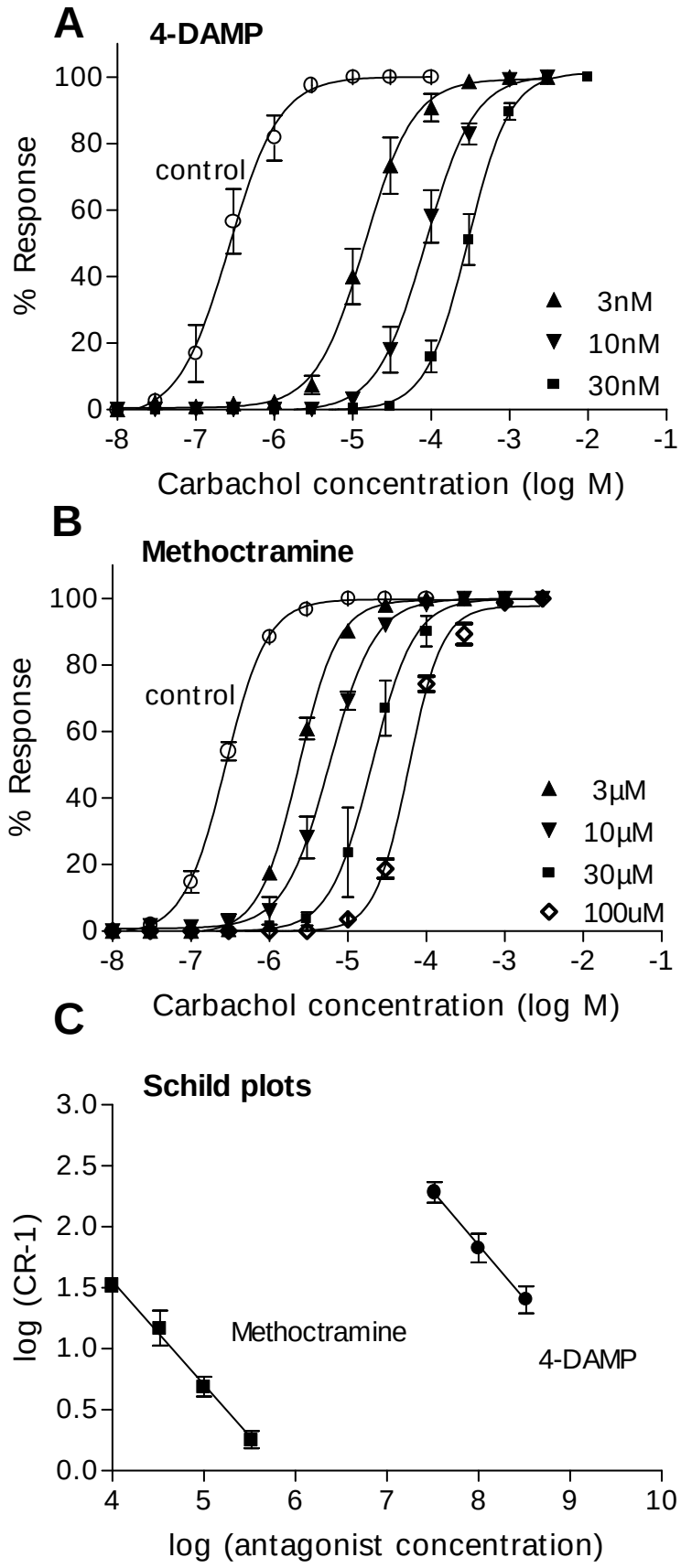
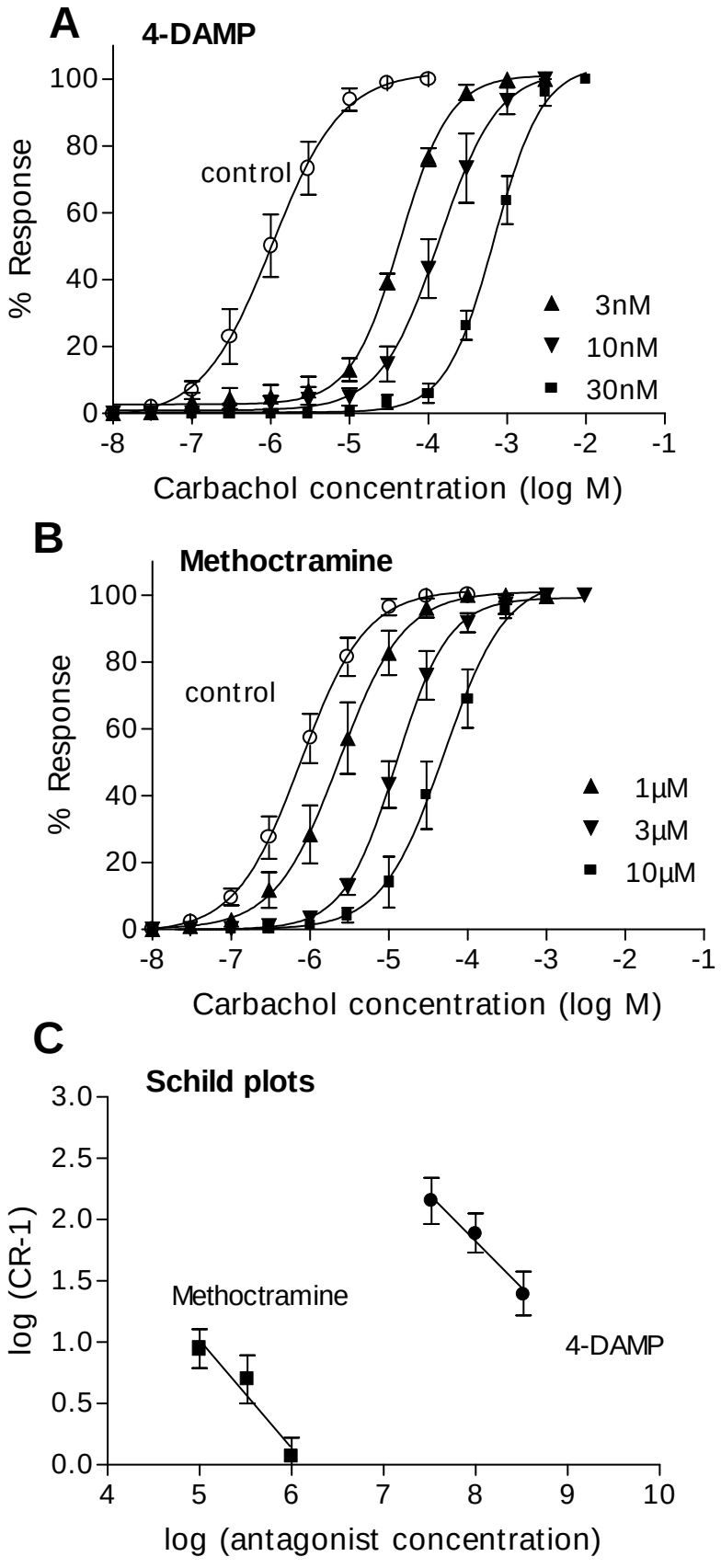

A
4-DAMP
% Response
control
3nM
10nM
30nM
Carbachol concentration (log M)
B
Methoctramine
% Response
control
3µM
10µM
30µM
100uM
Carbachol concentration (log M)
C
Schild plots
log (CR-1)
Methoctramine
4-DAMP
log (antagonist concentration)

Figure iv


A
4-DAMP
% Response
control
3nM
10nM
30nM
Carbachol concentration (log M)
B
Methoctramine
% Response
control
1µM
3µM
10µM
Carbachol concentration (log M)
C
Schild plots
log (CR-1)
Methoctramine
4-DAMP
log (antagonist concentration)

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