

## Role of muscarinic $M_2$ and $M_3$ receptors in guinea-pig trachea: effects of receptor alkylation

Nikki Watson <sup>\*</sup>, Helen Reddy, Richard M. Eglén

*Syntex Discovery Research, Institute of Pharmacology, 3401 Hillview Avenue, Palo Alto, CA 94303, USA*

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### Abstract

Muscarinic  $M_2$  receptors account for more than half the muscarinic receptor population in smooth muscles of a number of species and yet it is the smaller  $M_3$  receptor population that mediates contraction of many of these tissues. The role of the majority of  $M_2$  receptors in the control of smooth muscle tone is unclear. In guinea-pig ileal smooth muscle, an indirect contractile role (re-contraction) for  $M_2$  receptors has been demonstrated in tissues subjected to  $M_3$  receptor alkylation and stimulation of adenylyl cyclase. The present studies have employed the technique of irreversible receptor alkylation in order to investigate the role of muscarinic  $M_2$  and  $M_3$  receptors in the control of guinea-pig tracheal smooth muscle tone. Experiments were performed to determine (i) whether an indirect contractile role for  $M_2$  receptors can be demonstrated in tracheal smooth muscle as described for ileum, and (ii) whether stimulation of  $M_2$  receptors can inhibit isoprenaline-induced relaxations of histamine pre-contracted trachea after selective  $M_3$  receptor alkylation. Our results suggest (i) that there is no evidence of  $M_2$  receptor-mediated re-contraction of tracheal smooth muscle after  $M_3$  receptor alkylation and stimulation of adenylyl cyclase, but (ii) that activation of  $M_2$  receptors, after  $M_3$  receptor alkylation, has a small inhibitory effect on relaxant responses to isoprenaline in guinea-pig tracheal smooth muscle. Therefore, it appears that the major role of postjunctional muscarinic  $M_2$  receptors in guinea-pig trachea remains to be determined.

**Keywords:** Muscarinic receptor;  $\beta$ -Adrenoceptor; Receptor alkylation, irreversible

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### 1. Introduction

Activation of  $M_3$  receptors causes contraction of guinea-pig isolated trachea and ileum, even though approximately 70% of the muscarinic receptors present are of the  $M_2$  subtype (Ford et al., 1991; Haddad et al., 1991). The role of the predominant  $M_2$  receptor population in the control of smooth muscle tone is unclear. Muscarinic agonists inhibit adenylyl cyclase activity in a number of smooth muscles, including guinea-pig trachea and ileum (Pyne et al., 1992; Thomas et al., 1993; Reddy et al., 1995) via activation of  $M_2$  receptors (Zhang et al., 1991; Reddy et al., 1995). Since  $\beta$ -adrenoceptor relaxations of both of these tissues are mediated via stimulation of adenylyl cyclase activity,

$M_2$  receptor activation may inhibit  $\beta$ -adrenoceptor-mediated relaxations (Thomas et al., 1993; Watson and Eglén, 1994; Reddy et al., 1995). This has been demonstrated functionally in guinea-pig trachea as inhibition of relaxant responses to isoprenaline in tissues pre-contracted with muscarinic agonists (Watson and Eglén, 1994). Since contractile responses to muscarinic agonists in ileum are not sustained long enough to perform relaxation response curves to isoprenaline, functional demonstration of the inhibition of adenylyl cyclase by  $M_2$  receptors has taken a different approach. Thomas and colleagues (1993) and, more recently, Reddy et al. (1995), have shown that when  $M_3$  receptors are selectively alkylated in ileal smooth muscle, stimulation of  $M_2$  receptors causes an indirect contraction (re-contraction) by inhibiting the relaxant effect of isoprenaline on histamine-induced contractions.

The present work describes a series of studies using the technique of irreversible receptor alkylation to investigate the role of  $M_2$  and  $M_3$  receptors in control

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<sup>\*</sup> Corresponding author. Present address: Krankenhaus Grosshansdorf, Zentrum für Pneumologie und Thoraxchirurgie, LVA Hamburg, Wöhrendamm 80, D-22927 Grosshansdorf, Germany. Tel. (49)-41-02-601860, fax (49)-41-02-601866.

of guinea-pig tracheal smooth muscle tone. Selective alkylation of  $M_3$  receptors was achieved using 4-DAMP mustard (*N*-(2-chloroethyl)-4-piperidinyldiphenylacetate), a moderately  $M_3$  selective alkylating agent, in conjunction with  $M_2$  receptor protection by methoctramine to improve selectivity of the alkylation process. Methoctramine was used to protect  $M_2$  receptors, since this shows the highest  $M_2/M_3$  receptor selectivity of the muscarinic  $M_2$  antagonists available (Melchiorre et al., 1993). Our aims were to determine whether, after  $M_3$  receptor alkylation, (i) a contractile role for  $M_2$  receptors could be demonstrated, as has been reported in ileum of this species, and (ii) stimulation of  $M_2$  receptors could inhibit isoprenaline-induced relaxations of histamine pre-contracted trachea. Attempts to investigate the effect of selective  $M_2$  receptor alkylation on the relaxant potency of isoprenaline in (+)-*cis*-dioxolane pre-contracted tissues are also discussed.

A preliminary account of this work was presented to the British Pharmacological Society (Eglen et al., 1994).

## 2. Materials and methods

### 2.1. Tissue preparation

Male Dunkin Hartley guinea-pigs (250–350 g) were killed by  $CO_2$  asphyxiation. Tracheae were isolated and placed in oxygenated (95%  $O_2$ :5%  $CO_2$ ) modified Krebs solution (composition mM: KCl 4.6,  $KH_2PO_4$  1.2,  $MgSO_4$  1.2, NaCl 118.2, glucose 10.0,  $NaHCO_3$  24.8 and  $CaCl_2$  2.5) and cleaned of extraneous tissue. Tracheae were opened along their ventral surface and strip preparations were cut transversely, with each strip containing 3–4 cartilaginous rings. Silk (4-0) sutures were attached to the cartilaginous portions on either side of the smooth muscle bands and the preparations were suspended, at a resting tension of 1 g, in 10 ml organ baths containing aerated modified Krebs solution (pH 7.4, 37°C). Indomethacin (1  $\mu$ M) was present throughout, to inhibit prostaglandin synthesis. Tetrodotoxin (0.1  $\mu$ M) was present throughout, to eliminate possible pre-junctional effects of muscarinic agonists. Corticosterone (30  $\mu$ M) was present in studies with  $\beta$ -adrenoceptor agonists, to inhibit extraneuronal monoamine uptake. All preparations were allowed 60 min to equilibrate before beginning experimental protocols. Concentration-effect curves were performed in a cumulative manner using incremental concentrations spaced at half  $\log_{10}$  intervals.

### 2.2. Responses to oxotremorine M after $M_3$ receptor alkylation

The selective alkylating agent 4-DAMP mustard was used in conjunction with  $M_2$  receptor protection by methoctramine, to inactivate  $M_3$  receptors irreversibly

while leaving  $M_2$  receptors functionally intact. Cumulative concentration-effect curves to oxotremorine M were compared before and after alkylation. The highly efficacious, non-selective muscarinic agonist oxotremorine M was used for these studies, since this was the agonist of choice in the ileal studies (Thomas et al., 1993; Reddy et al., 1995). The nature of the receptor subtype mediating contractions after this procedure was investigated using 0.3  $\mu$ M *para*-fluoro-hexahydro-siladifenidol (*p*-F-HHSiD). These studies were performed to establish the degree of  $M_3$  receptor alkylation.

Concentration-effect curves to oxotremorine M were performed at the start of the experiment to establish control responses. Tissues were then washed and re-equilibrated in the presence of 1  $\mu$ M methoctramine for 20 min before and then during a 60 min exposure to 40 nM 4-DAMP mustard. After this time, tissues were washed twice at 10 min intervals in the presence of 1  $\mu$ M methoctramine, to wash out the 4-DAMP mustard while maintaining  $M_2$  receptor protection. After this, tissues were washed at 10 min intervals for a further 60 min to eliminate both the methoctramine and remaining 4-DAMP mustard. Following this, concentration-effect curves to oxotremorine M were repeated in the absence or presence of 0.3  $\mu$ M *p*-F-HHSiD.

### 2.3. Responses to oxotremorine M after $M_3$ receptor alkylation in the presence of histamine and isoprenaline

After selective alkylation of  $M_3$  receptors as described above, tissues were exposed to histamine (3  $\mu$ M) and isoprenaline (10 nM) before performing cumulative concentration-effect curves to oxotremorine M. The purpose of this was to provide the necessary conditions for activation of  $M_2$  receptors to cause an indirect contraction (re-contraction) by reversing the relaxant effect of isoprenaline on the histamine contracture (Thomas et al., 1993; Reddy et al., 1995). The nature of the receptor subtype mediating contractions after this procedure was investigated using 1  $\mu$ M methoctramine, at a concentration predicted to block 98% of  $M_2$  receptors (Melchiorre et al., 1993).

### 2.4. Responses to oxotremorine M in the presence of histamine and isoprenaline but absence of $M_3$ receptor alkylation

These studies were performed in tissues not exposed to 4-DAMP mustard, to establish the degree of functional antagonism of contractile responses to oxotremorine M by the exposure to histamine (3  $\mu$ M) and isoprenaline (10 nM). Tissues were treated exactly as above (Section 2.3) but were not exposed to 4-DAMP mustard.

### 2.5. Effect of oxotremorine M and histamine on the relaxant potency of isoprenaline

These studies were performed to verify that oxotremorine M was capable of activating  $M_2$  receptors in this system and thereby reducing the relaxant potency of isoprenaline in tissues pre-contracted with oxotremorine M as compared to histamine.

Concentration-effect curves to oxotremorine M and histamine were established to determine the maximum response and the concentration of each agonist required to increase isometric tension by approximately 3 g. Tissues were then washed and re-equilibrated for 60 min in the absence or presence of methoctramine (0.3  $\mu$ M). The resting tension was then raised to approximately 3 g by the addition of either oxotremorine M (1  $\mu$ M) or histamine (30  $\mu$ M) and, once a stable contracture was achieved, concentration-effect curves to isoprenaline (0.1 nM–1  $\mu$ M) were performed.

### 2.6. Effect of (+)-*cis*-dioxolane on the relaxant potency of isoprenaline after $M_3$ receptor alkylation

This study was performed to investigate the effect of selective activation of  $M_2$  receptors on the relaxant potency of isoprenaline in tissues pre-contracted with histamine. Since there are no subtype-selective muscarinic agonists, the non-selective agonist (+)-*cis*-dioxolane was used in preparations in which  $M_3$  receptors were inactivated by irreversible alkylation using 4-DAMP mustard.  $M_3$  receptor alkylation was verified in these preparations by the absence of contraction to (+)-*cis*-dioxolane (0.1  $\mu$ M). This concentration of (+)-*cis*-dioxolane (0.1  $\mu$ M) was shown previously to inhibit isoprenaline-induced relaxations via activation of  $M_2$  receptors (Watson and Eglen, 1994).

Concentration-effect curves to (+)-*cis*-dioxolane were obtained in all tissues prior to performing  $M_3$  receptor alkylation as outlined above. After the alkylation procedure tissues were equilibrated in the presence or absence of methoctramine (0.3  $\mu$ M), prior to increasing the isometric tension to approximately 3 g using histamine (30  $\mu$ M). Once a stable histamine contracture was achieved (+)-*cis*-dioxolane (0.1  $\mu$ M) was applied and cumulative concentration-effect curves to isoprenaline were performed.

### 2.7. Analysis of data

All results were recorded as changes in isometric tension (g). Contractile responses were normalized to the maximal contractile response to agonist in each tissue. Relaxation responses were expressed as a percentage of the isometric tension induced by the agonist before application of isoprenaline. Data were analysed by the relationship of Parker and Waud (1971), using a

non-linear iterative curve fitting procedure (Kaleidagraph, Synergy Software, Reading, PA 19606, USA). The potency ( $pD_2$ ) and maximal response were determined by this procedure and, from these, apparent antagonist affinities ( $pK_B$ ) were determined using the method of Furchgott (1972).

Statistical analysis of data was performed using paired and unpaired Student's *t*-tests where appropriate, with  $P < 0.05$  being considered significant. All values quoted are the mean  $\pm$  S.E.M. for at least 5 animals, unless otherwise stated.

### 2.8. Drugs and chemicals used

(+)-*cis*-Dioxolane (L-(+)-*cis*-2-methyl-4-trimethylammoniummethyl-1,3-dioxolane iodide, a 60 : 40 *cis* : *trans* mixture), methoctramine, *p*-F-HHSiD (*para*-fluoro-hexahydrosiladifenidol), 4-DAMP mustard (*N*-(2-chloroethyl)-4-piperidinyldiphenylacetate), oxotremorine M and histamine were obtained from Research Biochemicals (Natick, MA, USA). Tetrodotoxin, indomethacin, corticosterone, isoprenaline and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 4-DAMP mustard was acidified with dilute acetic acid following solubilization in distilled water. Indomethacin was prepared as a 1 mg ml<sup>-1</sup> solution in propylene glycol and solubilized by a brief period (2–3 min) of sonication. Corticosterone was prepared as a 0.1 M solution in dimethyl sulphoxide. Tetrodotoxin was prepared as a 1.0 mM solution in 0.01 M acetic acid. Ascorbic acid (22  $\mu$ M) was added to solutions of histamine and isoprenaline as an anti-oxidant and these solutions were kept on ice for the duration of the experiments.

## 3. Results

### 3.1. Responses to oxotremorine M after $M_3$ receptor alkylation

4-DAMP mustard was used in conjunction with 1  $\mu$ M methoctramine to selectively alkylate  $M_3$  receptors. Under these conditions there was a  $43 \pm 2$ -fold rightward shift in the concentration-effect curves to oxotremorine M with no significant decrease in the maximum response. *p*-F-HHSiD (0.3  $\mu$ M) caused a significant additional rightward shift in the concentration-effect curves to oxotremorine M with an apparent affinity ( $pK_B$ ) value of  $6.9 \pm 0.1$  (Table 1, Fig. 1).

### 3.2. Responses to oxotremorine M after $M_3$ receptor alkylation in the presence of histamine and isoprenaline

After selective alkylation of  $M_3$  receptors, tissues were contracted with histamine (3  $\mu$ M) and relaxed

Table 1

The potency ( $pD_2$ ) of oxotremorine M in guinea-pig trachea under control conditions and after exposure to histamine ( $3 \mu M$ ) and isoprenaline ( $10 nM$ ) in the absence and presence of  $M_3$  receptor alkylation by 4-DAMP mustard

Treatment conditions	Before treatment	After treatment	Ratio <sup>a</sup>
4-DAMP mustard	$7.41 \pm 0.04$	$5.77 \pm 0.04$	NA
4-DAMP mustard + <i>p</i> -F-HHSiD ( $0.3 \mu M$ )	$7.45 \pm 0.04$	$5.41 \pm 0.07$	NA
4-DAMP mustard + Hist + Iso	$7.33 \pm 0.02$	$6.01 \pm 0.02$	$0.97 \pm 0.02$
4-DAMP mustard + Hist + Iso + methoctramine ( $1 \mu M$ )	$7.33 \pm 0.06$	$5.97 \pm 0.08$	$0.73 \pm 0.07$
Histamine and isoprenaline	$7.48 \pm 0.05$	$7.25 \pm 0.07$	$1.03 \pm 0.02$
Histamine and isoprenaline + methoctramine ( $1 \mu M$ )	$7.49 \pm 0.03$	$7.49 \pm 0.10$	$0.94 \pm 0.04$

<sup>a</sup> The ratio of the relaxation response to isoprenaline ( $10 nM$ ) over the contractile response to histamine ( $3 \mu M$ ). Hist, histamine; Iso, isoprenaline; NA, not applicable, since these tissues were not exposed to histamine or isoprenaline. Values are the mean  $\pm$  S.E.M.,  $n = 6$  per treatment group. \*  $P < 0.05$ ; NS, not significant.

with isoprenaline ( $10 nM$ ) before performing cumulative concentration-effect curves to oxotremorine M. Under these conditions there was a  $21 \pm 1$ -fold rightward shift in the concentration-effect curves to oxotremorine M with no significant decrease in the maximum response. Methoctramine ( $1 \mu M$ ) caused no additional shift in the concentration-effect curves to oxotremorine M (Table 1, Fig. 2). There was a significant depression in the maximum response to oxotremorine M in tissues treated with methoctramine ( $1 \mu M$ ) when compared to pre-alkylation responses. Contractions induced by histamine were completely reversed by isoprenaline in control tissues but not in those treated with methoctramine (Table 1).

### 3.3. Responses to oxotremorine M in the absence of $M_3$ receptor alkylation but presence of histamine and isoprenaline

In tissues not exposed to 4-DAMP mustard, pre-contracting with histamine ( $3 \mu M$ ) and then maximally

relaxing with isoprenaline ( $10 nM$ ), prior to performing concentration-effect curves to oxotremorine M, did not significantly alter responses to this agonist. The presence of  $1 \mu M$  methoctramine had no significant effect on contractile responses to oxotremorine M under these conditions and contractions induced by histamine were completely reversed by isoprenaline in all tissues (Table 1).

### 3.4. Effect of oxotremorine M and histamine on the relaxant potency of isoprenaline

Oxotremorine M and histamine caused concentration-dependent contractions of trachea with potencies ( $pD_2$ ) of  $7.05 \pm 0.11$  and  $5.54 \pm 0.15$ , respectively. The relaxant potency of isoprenaline in tissues pre-contracted to approximately 3 g using  $30 \mu M$  histamine was greater than in tissues pre-contracted to the same level of isometric tension with  $1 \mu M$  oxotremorine M ( $pD_2 = 8.43 \pm 0.07$  and  $7.83 \pm 0.10$ , respectively).

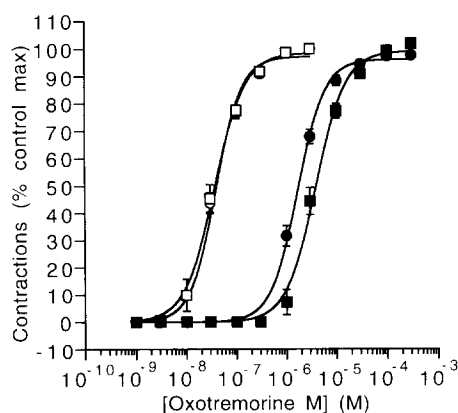


Fig. 1. Concentration-effect curves to oxotremorine M before (○ and □) and after (● and ■) selective  $M_3$  receptor alkylation in the absence (○, ● and □) and presence (■) of *p*-F-HHSiD ( $0.3 \mu M$ ). Contractions are expressed as a percentage of the maximum control response and are the mean  $\pm$  S.E.M.,  $n = 6$ .

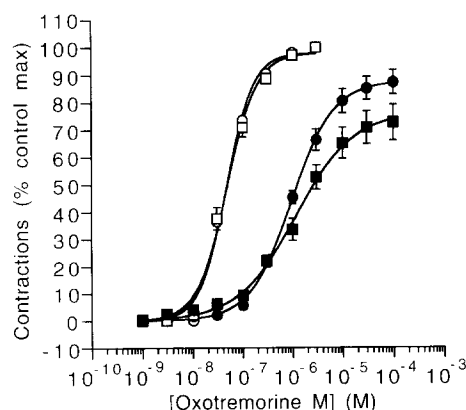


Fig. 2. Concentration-effect curves to oxotremorine M before  $M_3$  receptor alkylation (○ and □) and after selective  $M_3$  receptor alkylation in the presence of histamine and isoprenaline (● and ■) with (■) and without (●) methoctramine ( $1 \mu M$ ). Contractions are expressed as a percentage of the maximum control response and are the mean  $\pm$  S.E.M.,  $n = 6$ .

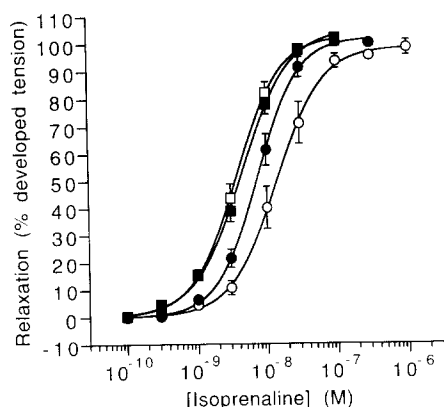


Fig. 3. Isoprenaline-induced relaxation of guinea-pig trachea contracted to approximately 3 g with oxotremorine M (circles) or histamine (squares) in the absence (open symbols) and presence (closed symbols) of 0.3  $\mu$ M methoctramine. Relaxations are expressed as a percentage of the developed tension induced by either oxotremorine M (1  $\mu$ M) or histamine (30  $\mu$ M). The relaxant potencies and levels of developed tension for all four groups are given in the text. Values are the mean  $\pm$  S.E.M.,  $n = 6$  per treatment group.

Methoctramine (0.3  $\mu$ M) had no significant effect on the relaxant potency of isoprenaline in tissues pre-contracted with histamine ( $pD_2 = 8.36 \pm 0.06$ ). However, in tissues pre-contracted with oxotremorine M, methoctramine (0.3  $\mu$ M) caused a significant 2-fold increase in the relaxant potency of isoprenaline ( $pD_2 = 8.11 \pm 0.06$ ) (Fig. 3).

There was no significant difference in the magnitude of the developed tension in any of these treatment groups prior to performing concentration-effect curves to isoprenaline ( $2.82 \pm 0.29$  g oxotremorine M alone,  $2.83 \pm 0.14$  g with methoctramine and  $2.88 \pm 0.11$  g histamine alone,  $2.70 \pm 0.16$  g with methoctramine).

### 3.5. Effect of (+)-cis-dioxolane on the relaxant potency of isoprenaline after $M_3$ receptor alkylation

After irreversible alkylation of  $M_3$  receptors using 4-DAMP mustard, all tissues were contracted to approximately 3 g with 30  $\mu$ M histamine. Addition of (+)-cis-dioxolane (0.1  $\mu$ M) caused no additional increase in isometric tension. However, the relaxant potency of isoprenaline was significantly reduced in tis-

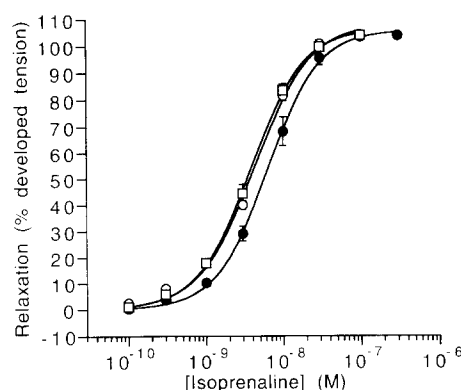


Fig. 4. Isoprenaline-induced relaxation of guinea-pig trachea, after selective  $M_3$  receptor alkylation, in tissues pre-contracted with histamine (30  $\mu$ M) under the following conditions; in the absence of (+)-cis-dioxolane or methoctramine ( $\circ$ ), in the presence of 0.1  $\mu$ M (+)-cis-dioxolane ( $\bullet$ ) and in the presence of 0.1  $\mu$ M (+)-cis-dioxolane and 0.3  $\mu$ M methoctramine ( $\square$ ). Relaxant potencies and levels of developed tension are given in Table 2. Values are the mean  $\pm$  S.E.M.,  $n = 6$  per treatment group.

sues pre-contracted with histamine and exposed to (+)-cis-dioxolane, when compared to tissues pre-contracted with histamine but not exposed to (+)-cis-dioxolane. This effect was reversed by 0.3  $\mu$ M methoctramine (Table 2, Fig. 4).

## 4. Discussion

The aim of the present studies was to investigate the role of  $M_2$  and  $M_3$  receptors in the control of guinea-pig tracheal smooth muscle tone. We have previously reported that (i) antagonism of  $M_2$  receptors increases the relaxant potency of isoprenaline in guinea-pig trachea pre-contracted with (+)-cis-dioxolane, and (ii) stimulation of  $M_2$  receptors in tissues pre-contracted with histamine reduces the relaxant potency of isoprenaline (Watson and Eglen, 1994). The lack of highly selective  $M_2$  receptor agonists or  $M_3$  receptor antagonists makes it difficult to draw firm conclusions with respect to the magnitude of the  $M_2$  effect in guinea-pig trachea from this earlier work. The present study has utilized the technique of irreversible receptor alkyla-

Table 2

The effect of (+)-cis-dioxolane (0.1  $\mu$ M) alone and in the presence of methoctramine (0.3  $\mu$ M) on the relaxant potency of isoprenaline in tissues pre-contracted to histamine (30  $\mu$ M) after selective  $M_3$  receptor alkylation by 4-DAMP mustard

	Control	With (+)-cis-dioxolane	With (+)-cis-dioxolane and methoctramine
Developed tension (g)	$2.94 \pm 0.18$	$3.06 \pm 0.24$	$2.62 \pm 0.15$
Potency ( $pD_2$ )	$8.37 \pm 0.02$	$8.20 \pm 0.06$	$8.43 \pm 0.04$
	NS		

Control tissues received neither (+)-cis-dioxolane nor methoctramine. In all three groups the developed tension was achieved by histamine (30  $\mu$ M) and addition of (+)-cis-dioxolane (0.1  $\mu$ M) had no significant effect on the developed tension. Values are the mean  $\pm$  S.E.M.,  $n = 6$  per treatment group. \*  $P < 0.05$ ; NS, not significant.

tion in an effort to overcome this problem and better characterize the role of muscarinic receptors in control of guinea-pig tracheal smooth muscle tone.

Selective  $M_3$  receptor alkylation has been used successfully in guinea-pig ileum to demonstrate an inhibitory role for  $M_2$  receptors on  $\beta$ -adrenoceptor function (Thomas et al., 1993; Reddy et al., 1995). After alkylation of a proportion of the  $M_3$  receptor population with 4-DAMP mustard, activation of  $M_2$  receptors by oxotremorine M inhibits isoprenaline-induced relaxations. This manifests itself as a re-contraction of ileum exposed to histamine and isoprenaline. This approach has been used in the present studies to investigate whether  $M_2$  receptor-mediated re-contraction occurs in trachea. Also, to follow previous work (Watson and Eglen, 1994), the effect of selective  $M_3$  receptor alkylation was investigated on the relaxant potency of isoprenaline.

The irreversible  $M_3$  receptor antagonist, 4-DAMP mustard (Barlow et al., 1990) was used in conjunction with  $M_2$  receptor antagonism by methoctramine (1  $\mu$ M) to alkylate  $M_3$  receptors selectively and irreversibly while leaving  $M_2$  receptors functionally intact.  $M_3$  receptor alkylation under these conditions caused approximately a 40-fold rightward shift in the concentration-effect curve to oxotremorine M with no depression in the maximum response. This is a greater shift than achieved in guinea-pig ileum under these conditions (20-fold shift, Thomas et al., 1993; 16-fold shift, Reddy et al., 1995). The reasons for this are unclear, but would be advantageous for observing  $M_2$  receptor-mediated re-contractions (Thomas et al., 1993; Reddy et al., 1995). Despite irreversible antagonism, by 4-DAMP mustard, of a large proportion of  $M_3$  receptors, the receptor reserve associated with contraction in trachea was maintained (Eglen and Whiting, 1988). In support of this, contractions to oxotremorine M were surmountably antagonised by *p*-F-HHSD, with an apparent affinity value (6.9) consistent with the activation of  $M_3$  receptors. This value is low when compared to  $M_3$  receptors in other smooth muscles, including guinea-pig ileum (7.8–8.0, Lambrecht et al., 1988), but consistent with previous findings in guinea-pig trachea (Eglen et al., 1990; Watson and Eglen, 1994).

To reveal  $M_2$  receptor-mediated re-contractions after  $M_3$  receptor alkylation, tissues were exposed to histamine and isoprenaline (Thomas et al., 1993; Reddy et al., 1995). Under these conditions the concentration-effect curve to oxotremorine M was shifted to the right 21-fold, but was not shifted further by  $M_2$  receptor antagonism using methoctramine. Therefore, it appears that these contractions were not mediated by  $M_2$  receptors and that  $M_3$  receptors probably mediated this response. There was a significant depression of the maximum response to oxotremorine M in tissues

treated with methoctramine (1  $\mu$ M) after exposure to 4-DAMP mustard. This concentration of methoctramine did not appear to have significant  $M_3$  receptor antagonist activity in control experiments (see below). The depression of maximum responses after alkylation may suggest that, with the  $M_3$  receptor reserve reduced by alkylation, further occupation of  $M_3$  receptors by methoctramine reduces the maximum contraction. However, in these tissues isoprenaline (10 nM) did not completely reverse the contraction induced by histamine (3  $\mu$ M). The reasons for this are unclear, but may also have contributed to the depression in maximum contraction to oxotremorine M.

In the absence of  $M_3$  receptor alkylation, contractile responses to oxotremorine M were unaltered by the presence of histamine and isoprenaline and there was no significant effect of the  $M_2$  receptor antagonist, methoctramine. These data suggest that there was no functional antagonism of contractile responses to oxotremorine M under these conditions of histamine and isoprenaline. It is interesting to note, therefore, that the rightward shift in responses to oxotremorine M after  $M_3$  receptor alkylation when histamine and isoprenaline were present was reduced (20-fold versus 40-fold). The reasons for this are unclear. It is possible that, under conditions of reduced functional  $M_3$  receptor number, stimulation of inositol phosphate production by histamine lowers the threshold for  $M_3$  receptor-mediated contractions.

Based on apparent antagonist affinity values ( $pK_B$ ) of 7.8 and 6.3 for methoctramine at  $M_2$  and  $M_3$  receptors, respectively (Melchiorre et al., 1993), 98% and 67% of these receptors were occupied by 1  $\mu$ M methoctramine, during the alkylation procedure. It might be argued that insufficient  $M_3$  receptor alkylation was achieved in these studies since a number of  $M_3$  receptors were occupied with this concentration of methoctramine. However, this is unlikely to be the case, since studies were also performed using 0.3  $\mu$ M methoctramine to protect  $M_2$  receptors during alkylation. Under these conditions 37%  $M_3$  receptor occupancy would be predicted and, as might be anticipated by such a change in alkylation conditions, the concentration-effect curves to oxotremorine M were further shifted to the right (approximately 100-fold) and exhibited a significant reduction in the maximum response, indicating a loss of receptor reserve for contraction. Despite these changes in alkylation conditions, no  $M_2$  receptor-mediated re-contraction could be detected after exposure to histamine and isoprenaline.

One explanation for the lack of an  $M_2$  receptor-mediated re-contraction in this tissue could be that oxotremorine M had a low efficacy and did not stimulate  $M_2$  receptor-mediated inhibition of isoprenaline relaxations (Watson and Eglen, 1994). To determine if this was the case, experiments were performed compar-

ing the relaxant potency of isoprenaline in tissues pre-contracted with oxotremorine M and histamine in both the absence and presence of methoctramine (0.3  $\mu$ M). These results suggest that this is unlikely to be the explanation for the lack of  $M_2$  receptor-mediated re-contraction, since (i) the relaxant potency of isoprenaline was significantly reduced in tissues pre-contracted with oxotremorine M when compared to histamine, and (ii)  $M_2$  receptor antagonism by methoctramine significantly increased the relaxant potency of isoprenaline in tissues pre-contracted with oxotremorine M but not with histamine. It is unlikely that  $M_3$  receptor antagonist properties of methoctramine account for the effect observed, since we have previously found this concentration (0.3  $\mu$ M) to be without significant  $M_3$  receptor antagonist activity in this tissue (Watson and Eglén, 1994). It should be noted that methoctramine did not increase the relaxant potency of isoprenaline to the same level as seen in tissues pre-contracted with histamine. This is consistent with previous observations using (+)-*cis*-dioxolane (Watson and Eglén, 1994) and suggest that activation of  $M_2$  receptors does not entirely explain the difference in relaxant potencies.

Attempts to stimulate  $M_2$  receptors in the absence of  $M_3$  receptor activation in order to investigate the influence of  $M_2$  receptors on isoprenaline relaxant potency have been reported previously (Watson and Eglén, 1994). These studies were hindered by the lack of selective  $M_3$  receptor antagonists. This difficulty has been overcome in the present study by using selective alkylation of  $M_3$  receptors with 4-DAMP mustard. Under these conditions, stimulation of  $M_2$  receptors using (+)-*cis*-dioxolane (0.1  $\mu$ M) caused a small but significant reduction in the relaxant potency of isoprenaline in histamine pre-contracted tissues. The reversal of this effect by methoctramine suggests that activation of  $M_2$  receptors, by (+)-*cis*-dioxolane, was responsible for the reduction in potency.

The findings from this study and our previous work (Watson and Eglén, 1994) suggest that, while activation of  $M_2$  receptors can be shown to reduce directly the relaxant potency of isoprenaline in guinea-pig trachea, the effect is small and does not appear to be demonstrable by the indirect approach used in ileal smooth muscle (Thomas et al., 1993; Reddy et al., 1995). Taken together these data suggest that activation of  $M_2$  receptors has only a minor contribution to the functional antagonism of  $\beta$ -adrenoceptor responses. A more physiologically significant role may be played by differences in the level of phosphoinositide metabolism induced by different spasmogens, as suggested by Zaagsma and colleagues (Van Amsterdam et al., 1989; Meurs et al., 1993). Therefore, it would appear that the major role of postjunctional muscarinic  $M_2$  receptors in guinea-pig trachea remains to be determined.

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