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Muscarinic M_2 Receptor-Mediated Contraction in the Guinea Pig Taenia Caeci

POSSIBLE INVOLVEMENT OF PROTEIN KINASE C

*Albert Shen and Fred Mitchelson**

VICTORIAN COLLEGE OF PHARMACY (MONASH UNIVERSITY), DEPARTMENT OF PHARMACEUTICAL BIOLOGY AND PHARMACOLOGY, PARKVILLE, VICTORIA 3052, AUSTRALIA

ABSTRACT. Contraction of the guinea pig taenia caeci is mediated by muscarinic M_3 receptors; however, they comprise only 30% of the muscarinic receptors present. This study investigated the role of the predominant M_2 receptor population in contractions and possible second messengers involved after M_3 receptors were selectively alkylated by 4-DAMP mustard [*N*-(2-chloroethyl)-4-piperidinyldiphenylacetate] (60 nM) in the presence of otenzepad (AF-DX 116; 1 μM). Concentration–response curves to oxotremorine-M (oxo-M) in the presence of histamine and isoprenaline were performed in the presence of otenzepad (1 and 3 μM), resulting in a mean apparent pK_B of 6.49, indicative of an M_2 response. As the taenia has intrinsic tone, precontraction with histamine was not necessary and, therefore, in some experiments only isoprenaline was included. In these studies, an M_3 response to oxo-M was observed, as the mean apparent pK_B for otenzepad was 5.89. To investigate protein kinase C (PKC) involvement in the M_2 response following M_3 inactivation, the inhibitor chelerythrine (1 μM) was included with histamine and isoprenaline in the absence and presence of otenzepad. The oxo-M concentration–response curve was shifted by otenzepad with an apparent pK_B value of 6.05, a value significantly different from that seen in the absence of chelerythrine ($P < 0.05$). These results suggest that activation of PKC by a spasmogen such as histamine is necessary to see an M_2 response following M_3 receptor inactivation. BIOCHEM PHARMACOL **56**;11:1529–1537, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. M_2 and M_3 muscarinic receptor subtypes; taenia caeci; smooth muscle contraction; protein kinase C

Molecular cloning techniques have established the existence of five cloned muscarinic acetylcholine receptor subtypes, m1–m5 [1–4]. The m1–m4 genes correspond pharmacologically to the defined M_1–M_4 receptors [5, 6]. The M_1 and M_3 receptors are coupled to G proteins of the $G_{q/11}$ family, which are insensitive to pertussis toxin [7, 8] and cause phosphatidylinositol hydrolysis, an increase in intracellular calcium and arachidonic acid levels, and opening of calcium-dependent ion channels [5, 9, 10]. The M_2 and M_4 receptors are coupled to G proteins of the $G_{i/o}$ class [11, 12], are sensitive to pertussis toxin, and cause inhibition of adenylyl cyclase and weak phosphatidylinositol hydrolysis [13].

Muscarinic receptors in smooth muscle of many tissues have been reported to be heterogenous. These tissues, including the guinea pig ileum [14], rat ileum [15], guinea pig uterus [16], and bovine trachea [17], contain a mixture of both M_2 and M_3 receptors. In general, 70–80% of receptors are of the M_2 subtype and 20–30% of the M_3 subtype. It has been observed that pertussis toxin treatment does not affect significantly contractions of the guinea pig ileum to muscarinic agonists [18], nor does inactivation of M_2 receptors by selective alkylation [19]. Thus, although the M_2 receptors are present in a greater proportion, it is the M_3 receptors that appear responsible for smooth muscle contraction in these tissues [5, 15, 20, 21], and the role of the M_2 receptor in contraction is speculative.

The M_2 receptor has been shown to inhibit basal adenylyl cyclase activity in smooth muscle [15, 22–24], as well as the increase in enzyme activity induced by β-receptor activation or by forskolin [23–25]. As Berridge [26] found that elevated cyclic AMP levels are able to relax smooth muscle, it has been suggested that M_2 receptors may be able to counter this relaxation by inhibiting the increase in cyclic AMP levels caused by such enzyme activation [15]. Recently it has been reported that M_2 receptors can couple to a cation channel, which causes depolarization and is potentiated by M_3 receptor activation [27, 28].

One method employed to study the M_2 receptor in the absence of M_3 receptor activity has been to selectively inactivate the latter with 4-DAMP mustard,† as used by

* Corresponding author: Dr. Fred Mitchelson, Victorian College of Pharmacy (Monash University), Department of Pharmaceutical Biology and Pharmacology, 381 Royal Parade, Parkville, Victoria 3052, Australia. Tel. 61-3-9903-9562; FAX 61-3-9903-9638; E-mail: Fred.mitchelson@vcp.monash.edu.au

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† *Abbreviations:* ACh, acetylcholine; 4-DAMP mustard, *N*-(2-chloroethyl)-4-piperidinyldiphenylacetate; oxo-M, oxotremorine-M; PG, prostaglandin; and PKC, protein kinase C.

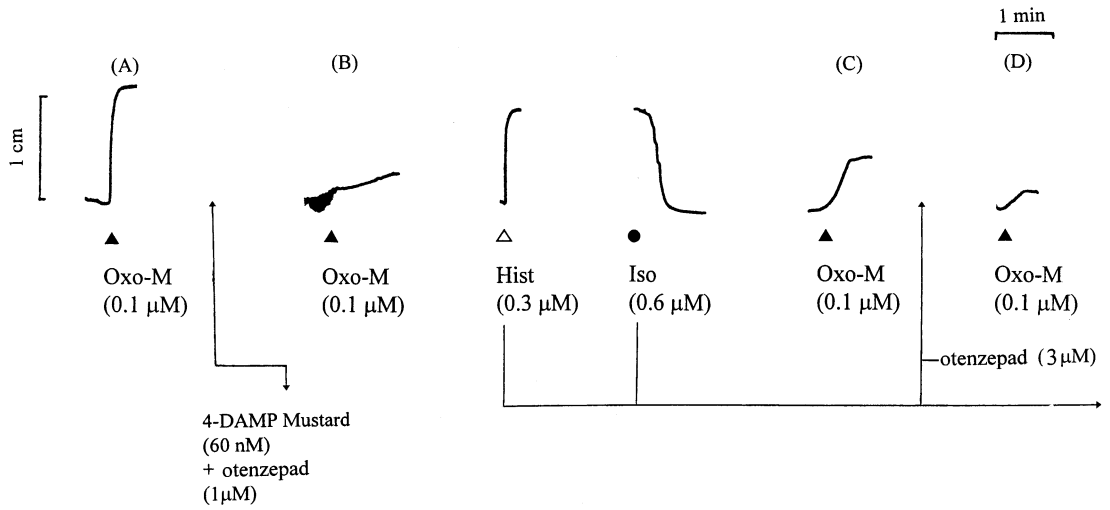

FIG. 1. Results from a single typical experiment to investigate M_2 receptors illustrated by the response to a single concentration of oxo-M (0.1 μM). (A) Control response to oxo-M in untreated tissue. (B) Response to oxo-M following alkylation of the M_3 receptor by 4-DAMP mustard while protecting the M_2 receptors with otenzepad. (C) Response to oxo-M following addition of histamine and isoprenaline. (D) Response to oxo-M following treatment with otenzepad in the continued presence of histamine and isoprenaline. In these experiments, several concentrations of oxo-M would be applied similarly and in duplicate to obtain a concentration–response curve. Note that there was little alteration in the tone of the preparation whenever oxo-M was added (▲).

Ehlert and co-workers in the guinea pig ileum [29]. This method involves receptor alkylation with 4-DAMP mustard in the presence of a selective M_2 receptor antagonist such as otenzepad (AF-DX 116), in order to protect the M_2 receptors from alkylation. Following prolonged washout, preparations are precontracted with histamine and relaxed to resting tension with isoprenaline before concentration–response curves to the agonist oxo-M are established. Using this protocol, in the guinea pig ileum, it was found that M_2 receptor activation was able to reverse the β-adrenoceptor-mediated relaxation, thereby indirectly causing a contraction [21, 29–33]. This method of receptor alkylation, followed by the addition of a spasmogen and a relaxing agent, has also been used in several other tissues to investigate M_2 receptor function, including the guinea pig trachea [34, 35], guinea pig oesophagus and rat fundus [35], rat oesophagus [36], and rat urinary bladder [37].

The aim of this study was to investigate the role of the M_2 receptor, using the above-mentioned method, in the contraction of the guinea pig taenia coli, another tissue that has a high proportion (70%) of M_2 receptors [38]. The taenia has intrinsic tone, and thus it is not necessary to employ histamine or some other spasmogen to raise the tone before use of a relaxing agent. Thus, this tissue allowed some evaluation of the contribution of the spasmogen in revealing the M_2 receptor contraction.

MATERIALS AND METHODS

Guinea pigs (200–400 g) of either sex were killed by a sharp blow to the back of the neck and exsanguinated from the carotid arteries. Lengths of taenia caeci (2 cm) were suspended under 0.5 g weight tension in a 10-mL organ bath containing Krebs' solution gassed with carbogen (95% O_2:5% CO_2). The composition of the Krebs' solution was (mM): NaCl, 113; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5. Responses were recorded isotonically by a UgoBasile 7006 isotonic transducer and Grass model 79D polygraph trace recorder.

Alkylation by 4-DAMP Mustard

In early experiments, 4-DAMP mustard (40 nM) in the presence of methoctramine (0.1 μM) was added to the bath for 80 min to alkylate M_3 receptors while protecting M_2 receptors. In an attempt to improve the degree of M_2 receptor responsiveness after alkylation, the concentration of 4-DAMP mustard was increased to 60 nM, and methoctramine was replaced by otenzepad (1 μM). Following alkylation, the tissues were washed at 10-min intervals for 80 min. In the majority of experiments, this latter procedure involving otenzepad was followed, with oxo-M employed as the agonist.

Histamine Precontraction

Following the extensive washing period after alkylation, tissues were relaxed with isoprenaline (0.6 μM) in the absence or presence of histamine (0.3 μM). Concentration–response curves to oxo-M were then obtained using 4–5 concentrations of agonist, with the agonist left in contact with the tissue for 30 sec followed by washout before addition of another concentration. The responses were obtained in duplicate in the absence and presence of otenzepad (1 and 3 μM) (Fig. 1). Experiments were also conducted using forskolin (0.5 μM) as the relaxing agent, in place of isoprenaline.

Similar experiments were also conducted on the guinea

TABLE 1. EC_{50} Values for oxo-M and concentration ratios in the presence of 4-DAMP mustard (60 nM), and following the addition of histamine (0.3 μM), isoprenaline (0.6 μM), and otenzepad (1 and 3 μM)

Treatment	EC_{50} (nM)	Concentration ratio	Apparent pK_B
Control	9.27 (7.39–11.6, 20)		
4-DAMP mustard	240 (170–330, 20)	25.9 (20.0–33.5, 20)	
+ Hist + iso	82.5 (62.1–110, 10)		
+ Hist + iso + otenzepad (1 μM)	363 (236–558, 4)	4.42 (2.75–7.13, 4)	6.53
+ Hist + iso + otenzepad (3 μM)	776 (470–1280, 6)	9.36 (6.45–13.6, 6)	6.45
+ Iso	315 (249–398, 10)		
+ Iso + otenzepad (1 μM)	806 (419–1550, 4)	1.87 (1.54–2.26, 4)	5.94
+ Iso + otenzepad (3 μM)	808 (705–926, 6)	3.11 (2.67–3.63, 6)	5.85

Values are geometric means (95% confidence limits, N) for 4–20 experiments.

pig ileum. Segments of ileum (2–3 cm long) were suspended isotonically in 10-mL organ baths containing Krebs' solution gassed with carbogen as for the taenia.

ACh Experiments

In the initial series of experiments, ACh was used as the agonist to construct concentration–response curves before and after alkylation with 4-DAMP mustard (40 nM) in the presence of methoctramine (0.1 μM).

Then tissues were washed as for the oxo-M experiments. Concentration–response curves to ACh were obtained following precontraction with histamine (0.3 μM) and relaxation with isoprenaline (1 μM), in the absence and presence of methoctramine (0.1 μM). Experiments were also conducted in the absence of histamine.

Second Messenger Studies

To investigate the role of PKC in M_2 receptor activation, the PKC inhibitor chelerythrine was used. Following alkylation of the M_3 receptors, and M_2 receptor protection with otenzepad, a concentration–response curve to oxo-M in the presence of both histamine and isoprenaline was performed in the absence and presence of chelerythrine (1 μM). Experiments were also performed where the concentration–response curve to oxo-M was constructed in the presence of chelerythrine, histamine, and isoprenaline in the absence and presence of otenzepad (3 μM).

M_3 Contraction

To assess the role of PKC in the contraction produced by M_3 activation, a concentration–response curve with oxo-M was performed in the presence and absence of chelerythrine (1 μM).

Statistics

All data are expressed as geometric means with 95% confidence limits given in parentheses. The EC_{50} values were estimated with the programme GraphPad Prism. Apparent pK_B values were calculated using the equation $pK_B = -\log([\text{antagonist}]/(DR - 1))$ [39], where DR is the dose ratio. Statistical evaluation was performed using Student's *t*-test with significance at the 5% level.

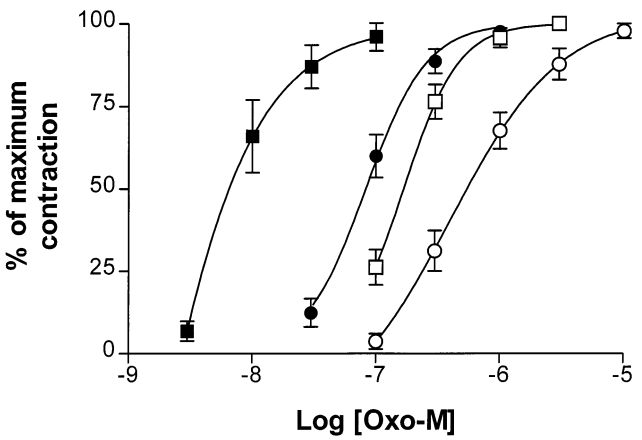

FIG. 2. Concentration–response curves for oxo-M in the absence (■) and presence (□) of 60 nM 4-DAMP mustard, then following histamine plus isoprenaline (●), and then in the presence of otenzepad (3 μM), histamine and isoprenaline (○) Data are means ± SEM, N = 6.

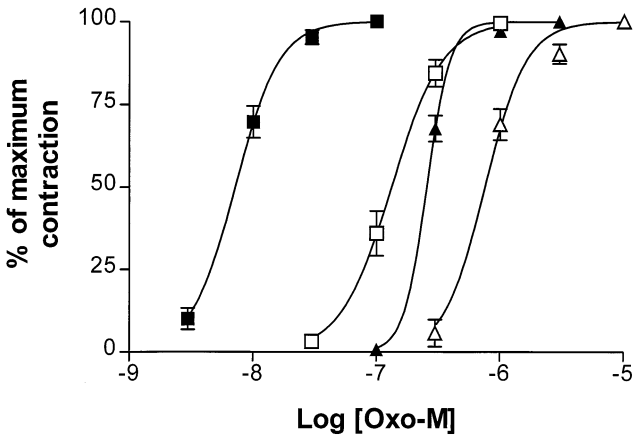

FIG. 3. Concentration–response curves for oxo-M in the absence (■) and presence (□) of 60 nM 4-DAMP mustard, then following isoprenaline only (▲) and then in the presence of otenzepad (3 μM) and isoprenaline (△). Data are means ± SEM, N = 6.

Drugs

Oxotremorine methiodide, 4-DAMP mustard hydrochloride, and chelerythrine chloride were obtained from Research Biochemicals Inc. Histamine diphosphate, ACh chloride, isoprenaline hydrochloride, and forskolin were obtained from the Sigma Chemical Co. Otenzepad (AF-DX 116) was donated by Dr. Karl Thomae, GmbH, and methoctramine was a gift from Dr. Carlo Melchiorre.

RESULTS

Responses to oxo-M

In untreated preparations, the concentration–response curve to oxo-M gave an EC_{50} of 9.27 nM (Table 1). Otenzepad (3 μM) caused a 4.46-fold shift in the oxo-M concentration–response curve with an apparent pK_B of 6.12 (3.92 to 6.93, 6) [geometric mean, (95% confidence limits, n)]. The addition of 4-DAMP mustard, with M_2 receptor protection, shifted the curve 25.9-fold (Table 1).

Histamine Precontraction Following Alkylation by 4-DAMP Mustard

Following alkylation of M_3 receptors and establishment of a concentration–response curve to oxo-M, addition of histamine plus isoprenaline produced a leftward shift of the oxo-M concentration–response curve with an EC_{50} of 82.5 nM (Table 1). Following addition of otenzepad (1 μM), the curve was shifted 4.42-fold with an apparent pK_B of 6.53. In experiments when 3 μM otenzepad was used, the dose ratio was 9.36 with an apparent pK_B of 6.45 (Table 1 and Fig. 2).

In experiments where histamine was omitted, there was no leftward shift of the oxo-M concentration–response curve (EC_{50} of 0.32 μM) in the presence of isoprenaline (Table 1 and Fig. 3), and dose ratios of 1.87 for 1 μM otenzepad and 3.11 for 3 μM otenzepad were obtained, yielding apparent pK_B values of 5.94 and 5.85, respectively (Table 1). These results were significantly different from those obtained in the presence of histamine ($P < 0.05$).

In experiments where forskolin (0.5 μM) was used in place of isoprenaline, in the presence of histamine and forskolin, the concentration–response curve to oxo-M was shifted 23.1-fold by otenzepad, giving an apparent pK_B of 6.87. In the absence of histamine, an apparent pK_B of 6.43 was obtained, which was significantly different ($P < 0.05$) from the value obtained in the presence of histamine (Table 2).

In studies using the guinea pig ileum, following 4-DAMP pretreatment, the concentration–response curve to oxo-M in the presence of histamine and isoprenaline was shifted 3.44-fold by otenzepad, yielding an apparent pK_B of 6.39 (Table 3).

TABLE 2. EC_{50} Values for oxo-M and concentration ratios in the presence of 4-DAMP mustard (60 nM), and following the addition of histamine (0.3 μM), forskolin (0.5 μM), and otenzepad (3 μM)

Treatment	EC_{50} (nM)	Concentration ratio	Apparent pK_B
Control	6.75 (4.27–10.7, 6)		
4-DAMP mustard	111 (86.3–141, 6)	14.7 (8.93–24.1, 3)	
+ Hist + forsk	43.0 (11.0–167, 3)		
+ Hist + forsk + otenzepad	993 (211–4680, 4)	23.1 (19.0–28.0, 3)	6.87
+ Forsk	276 (42.0–1804, 3)		
+ Forsk + otenzepad	2470 (330–18,500, 3)	9.0 (6.75–12.0, 3)	6.43

Values are geometric means (95% confidence limits, N) for 3–6 experiments.

TABLE 3. EC_{50} Values for oxo-M and concentration ratios in the guinea pig ileum following the addition of histamine (0.3 μM), isoprenaline (0.6 μM), and otenzepad (1 μM), following pretreatment with 4-DAMP mustard (60 nM)

Treatment	EC_{50} (nM)	Concentration ratio
Control	30.8 (20.1–47.1, 4)	
4-DAMP mustard	162 (114–231, 4)	5.24 (3.34–8.22, 4)
+ Hist + iso	202 (112–363, 4)	
+ Hist + iso + otenzepad	696 (305–1583, 4)	3.44 (1.85–6.39, 4)

Values are geometric means (95% confidence limits, N) for 4 experiments.

ACh Experiments

In the studies with the taenia caeci using ACh as the agonist, following 4-DAMP pretreatment in the presence of methoctramine, the concentration–response curve to ACh in the presence of histamine and isoprenaline was shifted 2.92-fold by methoctramine, giving an apparent pK_B of 7.28 (Table 4). In the absence of histamine, the results were found to be significantly different ($P < 0.05$), with the concentration–response curve being shifted only 1.51-fold, giving an apparent pK_B of 6.71 (Table 4).

Effect of Chelerythrine

The PKC inhibitor chelerythrine (1 μM) initiated a small degree of spontaneous activity in the tissue but did not raise the tone of preparations or affect contractions produced by oxo-M, histamine, or ACh (Table 5). Following M_3 inactivation with 4-DAMP, no change in the concentration–response curve to oxo-M in the presence of histamine and isoprenaline was observed, and upon addition of chelerythrine a dose ratio of 1.01 was found (data not shown). However, when chelerythrine was included along with histamine and isoprenaline, and a concentration–response curve was determined in the absence and presence of otenzepad (3 μM), a shift of 4.39 was seen, giving an apparent pK_B of 6.05 (Table 6). These results were significantly different ($P < 0.05$) from those obtained with histamine plus isoprenaline (Table 1).

DISCUSSION

The aim of this study was to investigate if M_2 receptors were involved in producing a contraction of the guinea pig taenia caeci, reversing the relaxant effect of the β-adrenoceptor agonist isoprenaline (a recontraction). The ability of M_2 receptors to produce this recontraction has been demonstrated in several tissues including guinea pig ileum [14, 29], rat ileum [23], rat bladder [37], canine trachea [25], guinea pig trachea [35], and rat oesophagus [36].

In the absence of any receptor inactivation, the interaction of otenzepad with M_3 receptors in the taenia was characterized by an apparent pK_B value of 6.12 when tested against the contractile response to oxo-M. Previous studies with otenzepad in this laboratory using carbachol- or McN-A-343-mediated contractions have yielded values of 5.6 to 5.8 [40], and a value of 5.9 was obtained by Takayanagi *et al.* [41] using carbachol.

M_2 Receptor Involvement

To examine M_2 receptor involvement without M_3 receptor activation, the M_3 receptors were selectively alkylated with 4-DAMP mustard, in the presence of otenzepad to protect the M_2 receptors. Following alkylation and then repeated washing, the concentration–response curve to oxo-M was shifted *ca.* 26-fold. In some preparations (*ca.* 41%), there was a 27.9 ± 0.04% depression of the maximal response to oxo-M, but not in the remainder. This finding suggests that

TABLE 4. EC_{50} Values for ACh and concentration ratios in the presence of histamine (0.3 μM), isoprenaline (0.6 μM), and methoctramine (0.1 μM) or isoprenaline alone and methoctramine, following pretreatment with 4-DAMP mustard (40 nM) and methoctramine (0.1 μM)

Treatment	EC_{50} (μM)	Concentration ratio	Apparent pK_B
Control	0.30 (0.13–0.69, 9)		
4-DAMP mustard	6.57 (3.73–11.7, 9)	21.9 (7.57–43.2, 9)	
+ Hist + iso	2.06 (1.09–3.81, 9)	0.31 (0.16–0.62, 9)	
+ Hist + iso + methoc	6.74 (3.31–17.2, 9)	2.92 (1.88–4.53, 9)	7.28
+ Iso	13.8 (9.12–20.9, 9)		
+ Iso + methoc	21.1 (13.0–33.6, 9)	1.51 (1.19–1.92, 9)	6.71

Values are geometric means (95% confidence limits, N) for 9 experiments.

TABLE 5. Effect of chelerythrine (1 μM) on concentration–response curves to oxo-M, histamine, and acetylcholine

	EC_{50} (nM)	
Agonist	**Control**	**Chelerythrine**
Oxo-M	14.2 (2.40–83.6, 3)	9.43 (5.53–16.1, 3)
Hist	125 (63.6–246, 8)	147 (96.5–224, 8)
ACh	220 (123–394, 8)	242 (136–434, 8)

Values are geometric means (95% confidence limits, N) for 3–8 experiments.

maximal responses to oxo-M can still occur with as little as 3.7% of the available M_3 receptor population. Similar findings for the taenia were observed with carbachol as the agonist, using propylbenzilylcholine mustard as the irreversible antagonist [42]. Although we did not characterize the response following the use of 4-DAMP mustard, such a response in the guinea pig ileum was characterized, using methoctramine, and was found to be mediated by M_3 receptors [30].

Following alkylation, and in the presence of histamine plus isoprenaline, otenzepad inhibited the concentration–response curve to oxo-M with a mean apparent pK_B of 6.49. Previous studies in this laboratory using otenzepad to antagonize oxotremorine-induced inhibition of adenylyl cyclase in the taenia caeci, an M_2 receptor-mediated response, found a pK_B of 6.95 [38]. Overall these results suggest that M_2 receptors were being activated after histamine and isoprenaline following 4-DAMP mustard pretreatment, although there may be a residual contribution from M_3 receptor activation that would account for the lower pK_B value obtained in this study compared with that where only an M_2 receptor was involved.

Using an identical protocol in guinea pig ileum with both histamine and isoprenaline present, the apparent pK_B for otenzepad was 6.39, similar to the value obtained in the taenia. Again, this was attributed to some residual M_3 receptor activation, although Thomas *et al.* [29] found a value of 6.77 for otenzepad in the guinea pig ileum with the same procedure.

Role of M_2 and M_3 Receptors

The abundance of M_2 receptors compared with M_3 receptors in various smooth muscle preparations has led to speculation of their role, as it is now established that M_3 receptors are involved in contractions of smooth muscle in the gut [15]. In this study, it appeared that the M_2 receptors were able to reverse the β-adrenoceptor-mediated relaxation (recontraction) after M_3 receptor inactivation. While this recontraction has been observed by others in many tissues of the rat and guinea pig, the significance of the M_2 receptor population varies. For example, in the rat fundus and guinea pig oesophagus, both M_2 and M_3 receptors are believed to contribute to the contractile response seen under conditions of elevated cyclic AMP levels [35], similar to the findings observed with the taenia caeci.

Role of Relaxing Agent

The relaxing agent used also appears to be crucial in determining the role of the M_2 receptor in producing contractions. M_2 receptors have been shown to facilitate oxo-M-induced contractions (M_3) in the presence of forskolin in untreated guinea pig trachea [35]. Other studies have shown similar results when using isoprenaline [25, 43]. In the guinea pig ileum, M_2 receptors have been reported to inhibit elevated cyclic AMP levels induced by PGE_1, PGE_2, serotonin, or vasoactive intestinal peptide [30]. Earlier studies by Griffen and Ehlert [23] observed attenuation of cyclic AMP levels induced by isoprenaline or forskolin but not those induced by PGE_1 or PGE_2. More recently,

TABLE 6. Effect of otenzepad (3 μM) on oxo-M responses in the presence of histamine (0.3 μM), isoprenaline (0.6 μM), and chelerythrine (1 μM), following 4-DAMP pretreatment (60 nM)

Treatment	EC_{50} (nM)	**Concentration ratio**	**Apparent pK_B**
+ Hist + iso + chel	70.3 (56.6–87.4, 5)		
+ Hist + iso + chel + otenzepad	308 (191–498, 5)	4.39 (3.05–6.31, 5)	6.05

Values are geometric means (95% confidence limits, N) for 5 experiments.

Ostrom and Ehlert [44] observed M_2 receptor-mediated inhibition of cyclic AMP levels induced by isoprenaline, forskolin, PGE_1, PGE_2, dopamine, serotonin, and vasoactive intestinal peptide, but no effect on cyclic AMP levels elevated by cicaprost, PGI_2, 5-methoxytryptamine, or dimaprit. In this study, when forskolin was used as the relaxing agent in the presence of histamine, following alkylation by 4-DAMP mustard, otenzepad caused a 23.1-fold shift of the concentration–response curve, resulting in an apparent pK_B of 6.87, indicative of an M_2 response (Table 2).

Preparations Studied in the Absence of Histamine

As the taenia has intrinsic tone, precontraction with histamine was not necessary, so experiments could be performed with only isoprenaline included. In these studies, following the addition of otenzepad, the oxo-M concentration–response curve was shifted with a mean apparent pK_B of 5.89, a result significantly different from that of experiments performed when histamine was present ($P < 0.05$). As noted earlier, a pK_B of this magnitude for otenzepad suggests only M_3 receptor involvement. When forskolin was used in place of isoprenaline, following otenzepad an apparent pK_B of 6.43 was observed, which was also significantly different from experiments where histamine was present ($P < 0.05$). Again, a 3- to 5-fold lower affinity of otenzepad for the receptor was obtained when histamine was absent.

Studies with ACh and Methoctramine

Another series of experiments were conducted where the agonist used was ACh and the M_2 receptor antagonist was methoctramine. In these experiments, only 40 nM 4-DAMP mustard was used but the shift of the EC_{50} for the agonist was comparable (21.9-fold) to that observed with oxo-M when 60 nM 4-DAMP mustard was used (25.9-fold). An apparent pK_B of 7.28 was observed with methoctramine in the presence of both histamine and isoprenaline, whereas in the presence of isoprenaline alone it was found to be 6.71. Again, the pK_B value in the presence of histamine was low for that of an M_2 receptor (7.6 to 7.9) [37, 45, 46], reinforcing the premise of some residual M_3 receptor involvement in this tissue. The result in the absence of histamine suggests that only M_3 receptors were involved (pK_B 6.1 to 6.7) [37, 45, 46]. In the taenia, differences have been noted in the ability of various muscarinic agonists to reduce cyclic AMP levels elevated by isoprenaline [38], so the finding that ACh caused contraction by muscarinic M_2 receptor activation is important since evidence that the endogenous cholinergic nerve transmitter is active supports a physiological role for the M_2 receptor.

It is interesting to note that following M_3 receptor inactivation by 4-DAMP mustard, re-establishment of the concentration–response curve to oxo-M in the presence of histamine and isoprenaline caused the EC_{50} to be shifted to the left, from 240 nM following 4-DAMP mustard, to 82.5 nM. This is in contrast to experiments with histamine absent, where the EC_{50} moved to the right (315 nM) (see Figs. 2 and 3). This was also the case in experiments where histamine and forskolin were used, compared with experiments where histamine was absent. That is, the EC_{50} was shifted from 111 nM following 4-DAMP mustard to 43 nM in the presence of histamine and forskolin, compared with 276 nM in the presence of forskolin alone. It may be that the lower EC_{50} value represents evidence of M_2 receptor activation occurring with a lower threshold than for residual M_3 receptor activation. A similar reduction in the EC_{50} has been noted in other tissues where M_2 receptor-induced recontraction has been obtained [29, 30, 35, 44].

Role of PKC

The results in this study discussed to date suggested that the presence of histamine was necessary to see an M_2 receptor response following M_3 receptor inactivation. The nature of the second messenger activated by histamine to fulfill this role was investigated. Chelerythrine is a selective PKC inhibitor. Herbert *et al.* [47] found that it inhibited PKC with an IC_{50} of 0.66 μM, with no effect on tyrosine kinase, cAMP dependent-protein kinase (PKA), or calcium/calmodulin-dependent protein kinase. Use of chelerythrine at a concentration of 1 μM was also found to have no effect on contractions due to oxo-M, ACh, or histamine. Following 4-DAMP mustard pretreatment, the addition of chelerythrine did not affect the oxo-M curve. However, when chelerythrine was included with histamine and isoprenaline, otenzepad was able to shift the oxo-M curve only 4.39-fold, giving a pK_B of 6.05, indicative of an M_3 response, which was significantly different from the mean value of 6.49 obtained in the presence of histamine plus isoprenaline alone. Thus, due to the fact that chelerythrine, a potent and selective PKC inhibitor, was able to reduce the pK_B of otenzepad to a value indicative of M_3 receptor stimulation, it would appear that activation of PKC is necessary for the M_2 receptor-mediated response following partial M_3 inactivation.

Concluding Remarks

Thus, the findings from the present study suggested that the presence of histamine is necessary for the cholinergic activation of the M_2 pathway following inactivation of a major fraction of the M_3 receptors in the guinea pig taenia caeci. Further, the findings with chelerythrine suggested that activation of PKC by histamine was a necessary step in this process. It may be speculated that activation of PKC by histamine substitutes for the normal modulation of the M_2 response by M_3 receptor-induced activation of this enzyme. That is, M_2 receptor activation may be involved in normal contraction but requires initial activation of M_3 receptors and, consequently, in the untreated smooth muscle tissue, antagonist pK_B values reflect this initial activation of the

M_3 receptor. After alkylation, there may be too few M_3 receptors remaining to allow concomitant activation of the M_2 receptor via the PKC-dependent mechanism. Consequently, histamine or some other spasmogen that activates PKC may be necessary to visualize involvement of the M_2 receptor. In other tissues, such as the cat oesophagus, a contraction may be directly mediated via M_2 receptors involving pertussis toxin-sensitive G_{13} proteins coupled to phosphatidylcholine-specific phospholipase D [48].

During the course of the current studies it was reported that muscarinic receptor activation by ACh or carbachol produces a cationic current and depolarization, resulting in smooth muscle contraction [49]. Pertussis toxin has been observed to abolish this current, indicating the possible involvement of the M_2 or M_4 muscarinic receptor. Recent findings indicate that the M_2 receptor mediates the cationic channel opening and that the M_3 receptor modulates this action [27, 28, 50]. It would be of interest to see if PKC was involved in the M_3 receptor-induced modulation of the M_2-mediated cation current, postulated by Bolton and Zholos [27].

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