British Journal of Pharmacology (2000) 129, 1458–1464

www.nature.com/bjp

Functional role of muscarinic M_2 receptors in α,β-methylene ATP induced, neurogenic contractions in guinea-pig ileum

[1]Gregory W. Sawyer, [2]Günter Lambrecht & *,[1]Frederick J. Ehlert

[1]Department of Pharmacology, College of Medicine, University of California, Irvine, California, CA 92697-4625, U.S.A. and [2]Department of Pharmacology, Biocentre Niederúrsel, University of Frankfurt, D-60439 Frankfurt/M Germany

1 The muscarinic acetylcholine receptors mediating the contractile response elicited to endogenous acetylcholine released by the selective P2X receptor agonist α,β-methylene ATP (mATP) were investigated in guinea-pig ileum.

2 mATP (0.1–30 μM) elicited a concentration-dependent neurogenic contractile response inhibited by tetrodotoxin (TTX) and antagonized by the non-selective muscarinic receptor antagonist N-methylscopolamine (NMS).

3 The contractile response to mATP was pertussis toxin-insensitive, irreversibly antagonized by N-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard), and unaffected by the muscarinic M_2/M_4 receptor selective antagonist AF-DX 116 (1 μM).

4 When measured in the presence of histamine and isoproterenol after treatment with 4-DAMP mustard, mATP elicited a pertussis toxin-sensitive contractile response potently antagonized by AF-DX 116.

5 Collectively, our data suggest that endogenous acetylcholine released by mATP can elicit a direct contractile response through the muscarinic M_3 receptor and an indirect contractile response through the muscarinic M_2 receptor by antagonizing the relaxant effects of isoproterenol on histamine induced contraction.

British Journal of Pharmacology (2000) **129,** 1458–1464

Keywords: Endogenous acetylcholine; muscarinic M_2 receptor; α,β-methylene ATP; α,β-methylene ATP and contraction; endogenous acetylcholine and contraction; pertussis toxin

Abbreviations: AF-DX 116, [[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one; 4-DAMP mustard, N-(2-chloroethyl)-4-piperidinyl diphenylacetate; KRB, Krebs-Ringer-bicarbonate buffer; mATP, α,β-methylene ATP; NMS, N-methylscopolamine

Introduction

In gastrointestinal smooth muscle, muscarinic receptor agonists are known to elicit contraction by acting on a mixed population of muscarinic M_2 and M_3 receptors (see reviews Eglen *et al*., 1996; Ehlert *et al*., 1997a,b). The muscarinic M_3 receptor is expressed at approximately one-quarter the density of the muscarinic M_2 receptor and mediates a pertussis toxin-insensitive contractile response to muscarinic agonists (e.g., oxotremorine-M) under standard assay conditions (i.e., in the absence of any other contractile or relaxant agent) (see reviews Eglen *et al*., 1996; Ehlert *et al*., 1997a). The M_3 receptor is known to couple with pertussis toxin-insensitive G_q proteins to mediate phosphoinositide hydrolysis and calcium mobilization. Presumably, mobilization of calcium initiates contraction.

Following M_3 receptor inactivation with 4-DAMP mustard, muscarinic agonists can elicit a highly potent, pertussis toxin-sensitive contractile response in guinea-pig ileum and colon provided that histamine and a cyclic AMP stimulating agent (e.g., forskolin or isoproterenol) are present (Thomas *et al*., 1993; Thomas & Ehlert, 1994; Ostrom & Ehlert, 1997; Sawyer & Ehlert, 1998). The M_2 receptor couples with pertussis toxin-sensitive G_i/G_o proteins and mediates an inhibition of adenylyl cyclase activity (Candell *et al*., 1990; Zhang & Buxton, 1991; Thomas & Ehlert, 1994) and cyclic AMP accumulation elicited by isoproterenol (Griffin & Ehlert, 1992; Ostrom & Ehlert, 1997; Sawyer & Ehlert, 1998) in gastrointestinal smooth muscle. Presumably, the contractile response measured in the presence of histamine and forskolin or isoproterenol is mediated by the M_2 receptor through an inhibition of cyclic AMP-mediated relaxation, enabling histamine to contract the muscle (see reviews Ehlert *et al*., 1997a,b). This experimental design allows one to observe a muscarinic M_2 mediated response in isolation from a muscarinic M_3 mediated response.

Transmural stimulation of the guinea-pig ileum has been demonstrated to release endogenous acetylcholine (Cowie *et al*., 1978; Kilbinger, 1982; Kilbinger *et al*., 1984). Endogenous acetylcholine, like exogenous oxotremorine-M, elicits a pertussis toxin-insensitive contractile response (Tucker, 1984; Lux & Schulz, 1986) mediated by the muscarinic M_3 receptor (Kilbinger *et al*., 1984; Kilbinger & Stein, 1988) under standard assay conditions. We have also shown that endogenous acetylcholine acts on muscarinic M_2 receptors when the cyclic AMP stimulating relaxant agents isoproterenol or forskolin are present (Sawyer & Ehlert, 1999) during transmural stimulation.

Purinergic agonists, acting on P2X-like receptors expressed on the soma-dendritic region of myenteric neurones, cause the release of endogenous acetylcholine and subsequent contraction of the guinea-pig ileum (Kennedy & Humphrey, 1994). Interestingly, these P2 receptors exhibit pharmacological properties distinct from those of recombinant P2Y and homomultimeric P2X receptors (Zhou & Galligan, 1996). Perhaps the P2 receptors in myenteric neurons might be heteropolymers of the cloned P2X receptor subunits or may contain additional P2X subunits not yet cloned (Ralevic & Burnstock, 1998; Lambrecht *et al*., 1999).

*Author for correspondence; e-mail: fjehlert@uci.edu

To examine the action of endogenous acetylcholine on muscarinic M_2 receptors in isolation from the M_3 receptor, we used the metabolically stable and selective P2X receptor agonist mATP (Ralevic & Burnstock, 1998) in the experimental paradigm described above (see Thomas *et al*., 1993). mATP has been shown to elicit contraction through the release of endogenous acetylcholine (Moody & Burnstock, 1982; Sperlagh & Vizi, 1991; Kennedy & Humphrey, 1994) acting on muscarinic M_3 receptors (Czeche *et al*., 1998; 1999). In the present study, we found that endogenous acetylcholine, released by exogenous mATP, acts on M_2 receptors in the guinea-pig ileum to elicit a pertussis toxin-sensitive contractile response when isoproterenol and histamine are present following selective inactivation of M_3 receptors.

Methods

In vivo *pertussis toxin treatment*

In some experiments, male Hartley guinea-pigs (250–300 g) were injected i.p. with 70 μg kg^{-1} body weight pertussis toxin, 3 days prior to being sacrificed.

Contractile measurements

Male Hartley guinea-pigs (250–350 g) were asphyxiated with CO_2 followed immediately by exsanguination. Segments of whole ileum (1.5–2.0 cm) were quickly dissected 1 cm proximal to the caecum and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB buffer mM): NaCl 124, KCl 5, $MgCl_2$ 1.3, $NaHCO_3$ 26, KH_2PO_4 1.2, $CaCl_2$ 1.8, glucose 10, gassed with O_2/CO_2, (19:1, v v^{-1}). Each segment was rapidly cleaned with KRB buffer to remove its contents, connected to a force transducer and mounted longitudinally in an organ bath containing 50 ml of KRB buffer at 37°C gassed with O_2/CO_2 (19:1). The ileal segments were allowed to equilibrate for 40 min at a resting tension equivalent to a load of 0.5 g prior to measuring isometric contractions with a force transducer and polygraph. A test dose of either histamine or mATP (a P2X receptor agonist) was then added to each bath. Once the tissue reached a sustained contraction, each bath was washed with KRB buffer and allowed to equilibrate 5 min prior to the addition of two more test doses. The test doses were used to ensure reproducibility of the preparations. Agonist concentration-response curves were measured using a total of 6–7 geometrically spaced (0.48 log units) concentrations of mATP. Following the addition of each concentration of mATP, the tissues were washed once with KRB (thus removing the mATP added) and allowed to incubate 6 min prior to the addition of the next concentration of mATP. The EC_{50} value was determined from this curve as described below. Following the addition of the final mATP concentration, the ileal segments were washed three times and allowed to incubate for 30 min before any additional measurements were made.

Some ileal segments were incubated with the aziridinium ion of N-(2-chloroethyl)-4-piperidinyldiphenylacetate (4-DAMP mustard; 40 nM) for 1 h in the presence of [[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido [2,3b] [1,4]benzodiazepine-6-one (AF-DX 116; 1.0 μM) to alkylate muscarinic M_3 receptors selectively (Thomas *et al*., 1993). The ileal segments were then washed thoroughly to remove AF-DX 116 and any unreacted 4-DAMP mustard. In all experiments, 4-DAMP mustard was first converted to its aziridinium ion by incubation for 30 min at 37°C in 10 mM $NaKPO_4$, pH 7.4, as previously described (Thomas *et al*., 1992).

Some 4-DAMP mustard-treated ileal segments were contracted with 0.64 μM histamine and relaxed with 0.32 μM isoproterenol prior to the addition of mATP (with both histamine and isoproterenol still present). In these experiments, the KRB was supplemented with physostigmine (70 nM) to inhibit cholinesterase activity.

Data analysis

The concentration of mATP eliciting half-maximal contraction (EC_{50}) was estimated by nonlinear regression analysis according to an increasing logistic equation as described previously (Candel *et al*., 1990).

To measure the inhibitory effect of 4-DAMP mustard on the contractions elicited to mATP, we used a procedure based on the principles of Furchgott analysis (Furchgott, 1966). We have previously used this approach in smooth muscle to analyse the concentration-response curve of a muscarinic agonist before and after partial receptor inactivation with 4-DAMP mustard. However, in this study, we have not measured responses to an exogenously applied muscarinic agonist, but rather, to mATP which elicits a contraction through the release of endogenous acetylcholine. If we assume that the concentration of acetylcholine (A) in the immediate vicinity of the muscarinic receptor can be described as a function (f) of the concentration of mATP (x), then the contractile responses (E) to mATP can be described by the following operational model of Black & Leff (1983):

$$E = E_m\tau^n[f(x)^n]/((K_A + [f(x)])^n + \tau^n[f(x)^n]) \qquad (1)$$

In this equation, E_m denotes the maximum possible response of the tissue, K_A denotes the equilibrium dissociation constant of acetylcholine, n denotes the coefficient of co-operativity in the stimulus-response function, and τ is equivalent to the expression $R_o\epsilon/\beta$, in which R_o denotes the receptor concentration, ϵ denotes intrinsic efficacy and β denotes the sensitivity constant in the stimulus-response function. A detailed explanation of this model is given by Black & Leff (1983) and Black *et al*. (1985). Equation [1] is equivalent to that described by Black *et al*. (1985) except that the agonist concentration has been replaced by the function $f(x)$. Since it would be difficult to determine (f), we made the provisional assumption that the concentration of acetylcholine is proportional to that of mATP (i.e., $A = cx$, in which c represents a constant). We fitted the mATP concentration-response curve to equation [1] by nonlinear regression analysis substituting the molar concentration of mATP for $f(x)$. We fitted the curves measured before and after partial receptor inactivation simultaneously sharing the same estimates of n, E_m and K_A for both curves and allowing the estimate of τ to vary between curves. By dividing the estimate of τ obtained after receptor inactivation (τ') with that measured before (τ''), the proportion of receptors not alkylated by 4-DAMP mustard is obtained. When analysed in this manner, the actual units of K_A are molar divided by the unknown constant c; consequently, we did not report the K_A value for endogenous acetylcholine. During the regression analysis, we fixed n at a value equal to that (2.1) measured in previous work on the guinea-pig ileum (Ehlert *et al*., 1999). The validity of our provisional assumption, that the endogenous acetylcholine concentration is proportional to the concentration of mATP, is evaluated under Discussion. Data are presented as mean ± s.e.mean from 3–4 observations.

Materials

The drugs and chemicals used in this investigation were obtained from the following sources: islet-activating protein (pertussis toxin), LIST Biological Laboratories, Campbell, CA, U.S.A.; AF-DX 116, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, U.S.A.; 4-DAMP mustard was synthesized in our laboratory as described previously (Thomas *et al*., 1992); and all remaining drugs and chemicals, Sigma Chemical Company (St. Louis, MO, U.S.A.).

Results

P2X receptors are known to be expressed on the soma-dendritic region of myenteric neurons of the guinea-pig ileum. mATP is a metabolically stable and selective P2X receptor agonist that mediates the release of acetylcholine from myenteric neurons resulting in a neurogenic contractile response (Moody & Burnstock, 1982; Kennedy & Humphrey, 1994; Bartho *et al*., 1997; Czeche *et al*., 1998; 1999; Lambrecht *et al*., 1999). Consistent with this observation, the contractile response of the guinea-pig ileum elicited to single concentrations of mATP was completely inhibited by tetrodotoxin (1 μM, data not shown). Tetrodotoxin is known to block sodium channels in a use dependent manner resulting in the blockade of neuronal transmission (Cohen *et al*., 1981). Therefore, the contractile response is neurogenic in nature rather than a direct action of mATP on postjunctional receptors.

To corroborate the observation that the contractile response elicited to mATP is a result of cholinergic neurotransmission, ileal segments were incubated with the non-selective muscarinic antagonist NMS 1 h prior to measuring concentration-response-curves to mATP. NMS (10 nM) caused a 1.7 fold increase in the EC_{50} value and a 75% decrease in the maximal contractile response elicited to mATP (Figure 1, Table 1). The large inhibitory effect of NMS suggests that the contractile response to mATP is a result of released acetylcholine acting on postjunctional muscarinic acetylcholine receptors.

Ileal segments were incubated with the aziridinium ion of 4-DAMP mustard (40 nM), in the presence of AF-DX 116 (1 μM), for 1 h to investigate the contribution of the muscarinic M_3 receptor to the contractile response elicited to mATP. The aziridinium ion of 4-DAMP mustard has been shown to be an irreversible, muscarinic receptor antagonist that alkylates muscarinic M_3 receptors selectively over muscarinic M_2 receptors, particularly when AF-DX 116 is present during the alkylation procedure (Thomas *et al*., 1992). AF-DX 116 is used to protect muscarinic M_2 receptors from alkylation by 4-DAMP mustard. One 4-DAMP mustard-treatment caused a 2.4 fold increase in the EC_{50} value and a 58% reduction in the maximal contractile response elicited to mATP (Figure 2, Table 1). The effect of 1 h 4-DAMP mustard-treatment corresponded to 66.6% receptor alkylation as estimated by the procedure outlined under Methods. The large inhibitory effect of 4-DAMP mustard suggests that the muscarinic M_3 receptor mediates the contractile response elicited to mATP under standard conditions. This observation is consistent with the pharmacological profile of the response as described by Czeche *et al*. (1998; 1999).

Table 1 The effects of NMS, AF-DX 116, 4-DAMP mustard, and pertussis toxin on the contractile response to mATP in the guinea-pig lieum

Conditions	*pEC_{50}*	*Maximal response*[a] (%)	*Occupancy*[b] (%)
Standard conditions			
Control	5.81±0.20	100	
NMS (10 nM)	5.76±0.17	25	67
4-DAMP mustard[c]	5.38±0.22	42	67
AF-DX 116 (1 μM)	5.91±0.21	95	~0
Pertussis toxin	5.68±0.37	104	
4-DAMP mustard-treated + histamine and isoproterenol[d]			
Control	5.59±0.22	100	
NMS (10 nM)	5.23±0.11	14	87
AF-DX 116 (1 μM)	4.93±0.47	49	71
Pertussis toxin	5.52±0.23	11	

[a]The EC_{50} and maximal response values of mATP were calculated as described under Methods. [b]Antagonist receptor occupancy was calculated as described under Discussion. [c]Guinea-pig ileum was incubated with 4-DAMP mustard (40 nM for 1 h in the presence of AF-DX 116 (1 μM) and washed extensively). [d]Guinea-pig ileum was incubated with 4-DAMP mustard (40 nM) for 1 h in the presence of AF-DX 116 (1 μM) washed extensively, and contractions to mATP were measured in the presence of histamine (0.32 μM) and isoproterenol (0.64 μM).

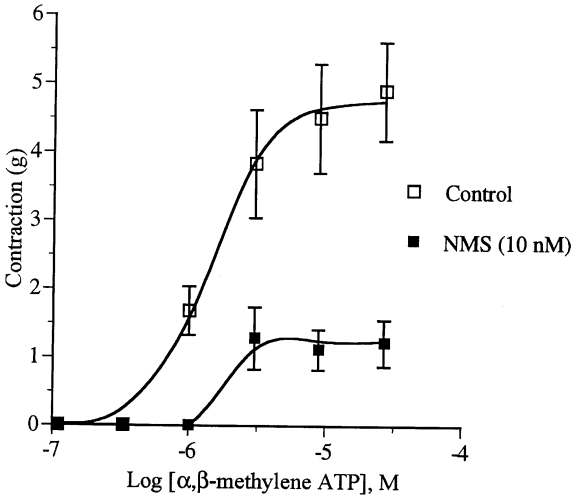

Figure 1 Effects of NMS on the contractile response elicited to mATP. Contractions were measured in the guinea-pig ileum in the absence and presence of NMS (10 nM). Each data point represents the mean±s.e.mean of three experiments.

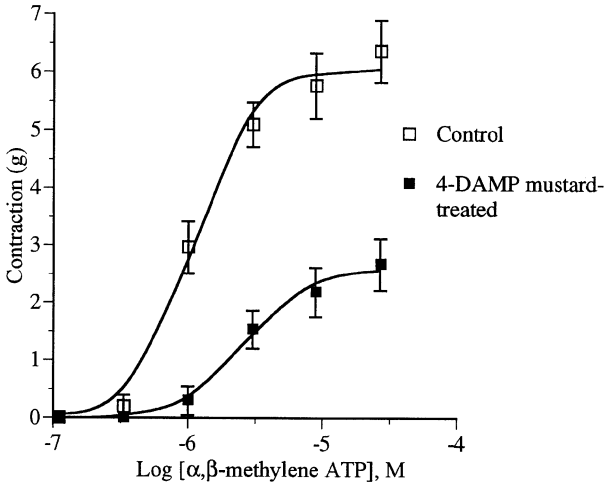

Figure 2 Effects of 4-DAMP mustard-treatment on the contractile response to mATP under standard conditions. Contractile response was measured in guinea-pig ileum before and after treatment with 4-DAMP mustard (40 nM) and AF-DX 116 (1 μM) for 1 h. Each data point represents the mean±s.e.mean of four experiments.

We investigated the effect of pertussis toxin on the standard contractile response of the guinea-pig ileum to mATP to determine whether the muscarinic M_2 receptor is contributing to the contraction under these conditions. Pertussis toxin is known to catalyze the ADP-ribosylation of G_i and G_o proteins resulting in the uncoupling of M_2- and M_4-mediated responses, without affecting M_1-, M_3-, and M_5-mediated responses (Kurise & Ui, 1983; Peralta *et al*., 1988). Pertussis toxin-treatment had no significant effect on the contractile response elicited to mATP (Table 1), suggesting that the muscarinic M_2 receptor does not contribute to the contractile response elicited to mATP under standard assay conditions.

When present at a concentration of 1 μM, the M_2/M_4 selective competitive antagonist AF-DX 116 was without effect on the contractile response elicited to mATP (Table 1) under standard assay conditions. Using the binding affinities for AF-DX 116 that our laboratory has previously estimated for the cloned human muscarinic M_2 ($pK_D = 7.27$) and M_3 ($pK_D = 6.10$) receptors in HEPES buffered KRB (Esqueda *et al*., 1996), we estimate that AF-DX 116 (1.0 μM) should cause 19.6- and 2.3 fold shifts in muscarinic M_2 and M_3 receptor mediated responses, respectively. Therefore, the lack of effect of AF-DX 116 on the standard contractile response to mATP in the guinea-pig ileum is nearly consistent with that expected for antagonism of a muscarinic M_3 receptor-mediated response. This observation supports the prior observation that the muscarinic M_2 receptor does not contribute to the contractile response elicited to mATP.

To investigate whether endogenous acetylcholine released by mATP also acts on muscarinic M_2 receptors, we used a protocol specifically designed for measuring the contractile effects of M_2 receptor activation (see Thomas *et al*., 1993). Ileal segments were treated with 4-DAMP mustard (40 nM) in the presence of AF-DX 116 (1 μM) for 1 h to inactive M_3 receptors. Thomas *et al*. (1993); Thomas & Ehlert (1994) have previously shown in the guinea-pig ileum that the contractile response elicited to oxotremorine-M following 4-DAMP mustard-treatment, and in the presence of histamine and isoproterenol, has a pharmacological profile consistent with the muscarinic M_2 receptor. Following 1 h 4-DAMP mustard-treatment and in the continued presence of histamine (0.32 μM) and isoproterenol (0.64 μM), mATP (20 μM) elicited a contractile response of 1.1 g (data not shown). We subsequently added physostigmine (70 nM) to the baths in the remainder of the experiments described below to increase the magnitude of the contractile response (Figures 3–5).

Following 1 h 4-DAMP mustard-treatment and in the continued presence of histamine (0.32 μM), isoproterenol (0.64 μM), and physostigmine (70 nM), mATP elicited a contractile response potently antagonized by NMS and AF-DX 116. NMS (10 nM) caused a 3.2 fold increase in the EC_{50} value and 86.5% decrease in the maximal response (Figure 3, Table 1), whereas AF-DX 116 (1 μM) caused a 2.62 fold increase in EC_{50} value and a 51% decrease in the maximal response (Figure 4, Table 1). The large inhibitory effect of AF-DX 116 suggests that the muscarinic M_2 receptor contributes to the contractile response elicited to mATP following 4-DAMP mustard-treatment and in the presence of histamine and isoproterol.

To investigate the role of the muscarinic M_2 receptor further, we measured contractile responses to mATP under the conditions just described in ileal segments harvested from pertussis toxin-treated guinea-pigs. Pertussis toxin-treatment caused a 1.47 fold increase in EC_{50} value and an 88.9% reduction in the maximal contractile response elicited to mATP when measured following 4-DAMP mustard-treatment and in the presence of histamine, isoproterenol, and physostigmine (Figure 5, Table 1). This large effect of pertussis toxin contrasts with the lack of effect observed in experiments on the standard contractile response to mATP (Table 1). These data suggest that the muscarinic M_2 receptor mediates the contractile response elicited to mATP following 4-DAMP mustard treatment and in the presence of histamine and isoproterenol.

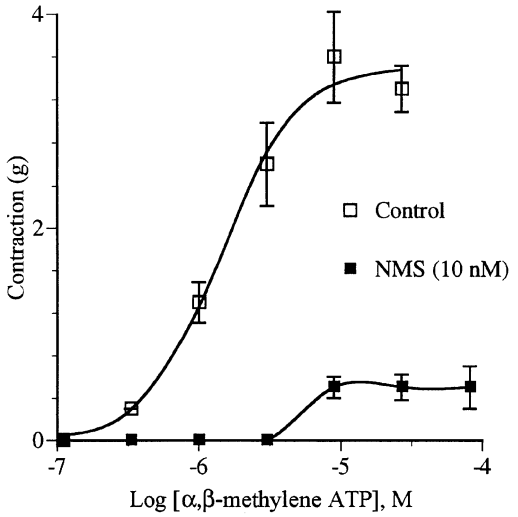

Figure 3 Effects of NMS on the contractile response to mATP measured in the presence of histamine and isoproterenol after 4-DAMP mustard-treatment. Following treatment with 4-DAMP mustard (40 nM) and AF-DX 116 (1 μM) for 1 h and extensive washing, ileal segments were contracted with histamine (0.32 μM) and relaxed with isoproterenol (0.64 μM) before measuring contraction to mATP in the absence and presence of NMS (10 nM). Each data point represents the mean ± s.e.mean of three experiments.

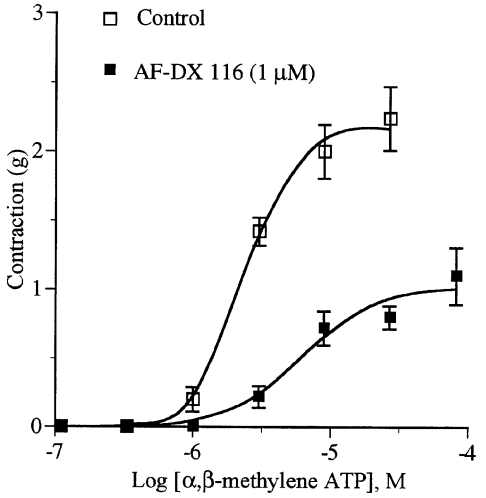

Figure 4 Effects of AF-DX 116 on the contractile response to mATP measured in the presence of histamine and isoproterenol after 4-DAMP mustard-treatment. Following treatment with 4-DAMP mustard (40 nM) and AF-DX 116 (1 μM) for 1 h followed by washing, ileal segments were contracted with histamine (0.32 μM) and relaxed with isoproterenol (0.64 μM) before measuring contraction to mATP in the absence and presence of AF-DX 116 (1 μM). Each data point represents the mean ± s.e.mean of four experiments.

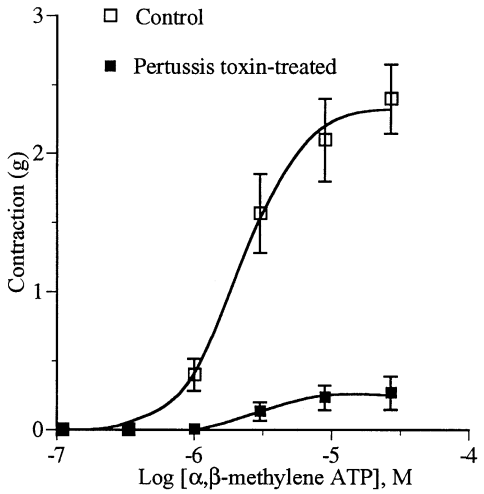

Figure 5 Effects of pertussis toxin-treatment on the contractile response to mATP measured in the presence of histamine and isoproterenol after 4-DAMP mustard-treatment. Following treatment with 4-DAMP mustard (40 nM) and AF-DX 116 (1 μM) for 1 h and extensive washing, ileal segments were contracted with histamine (0.32 μM) and relaxed with isoproterenol (0.64 μM) before measuring contraction to mATP in untreated and pertussis toxin-treated guinea-pig ileum. Each data point represents the mean $\pm$ s.e.mean of four experiments.

Discussion

The guinea-pig ileum expresses both muscarinic M_2 and M_3 receptors (see reviews Ehlert *et al*., 1997a,b). A large body of pharmacological evidence shows that the muscarinic M_3 receptor mediates the contractile response elicited to muscarinic agonists (see reviews Eglen *et al*., 1996; Ehlert *et al*., 1997a,b), including endogenous acetylcholine (Kilbinger *et al*., 1984; Tucker, 1984). In the present study, mATP was used to elicit contraction *via* release of acetylcholine from the myenteric plexus of the guinea-pig ileum. mATP has been shown previously to elicit a neurogenic contractile response mediated by acetylcholine (Kennedy & Humphrey, 1994) acting on the muscarinic M_3 receptor (Czeche *et al*., 1998; 1999). In accord with these prior studies, we found that mATP elicited a tetrodotoxin-sensitive contractile response inhibited by the nonselective muscarinic antagonists NMS (Figure 1).

The contractile response elicited to mATP was irreversibly antagonized by 4-DAMP mustard (Figure 2), weakly antagonized by the muscarinic M_2/M_4 selective antagonist AF-DX 116, and was unaffected by pertussis toxin-treatment (Table 1) consistent with a muscarinic M_3 receptor mediated event. This postulate supports the prior conclusion of Czeche *et al*. (1998; 1999) and is consistent with the well known role of the muscarinic M_3 receptor in mediating the contractile response of gastrointestinal smooth muscle.

In the smooth muscle of the guinea-pig ileum, most responses elicited to muscarinic receptor agonists can be characterized as being pertussis toxin-sensitive and potently antagonized by AF-DX 116 or pertussis toxin-insensitive and weakly antagonized by AF-DX 116. Muscarinic M_2 receptor mediated responses, like the inhibition of adenylyl cyclase activity, are potently antagonized by AF-DX 116 and inhibited by pertussis toxin (Candell *et al*., 1990; Griffin & Ehlert, 1992; Thomas *et al*., 1993). Conversely, muscarinic M_3 receptor mediated responses, like the contractile response and phosphoinositide hydrolysis, are weakly antagonized by AF-DX 116 and are not inhibited by pertussis toxin (see reviews Eglen *et al*., 1996; Ehlert *et al*., 1997a,b). Thus, the pharmacological profile of the contractile response elicited to mATP following 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol is consistent with a muscarinic M_2 receptor mediated event in that it is pertussis toxin-sensitive (Figure 5) and potently antagonized by AF-DX 116 (Figure 4). We have previously used this experimental paradigm to examine the effects of the muscarinic agonist oxotremorine-M instead of mATP. In these previous studies, we investigated the sensitivity of the contractile response to pertussis toxin as well as a variety of subtype selective muscarinic antagonists and obtained results consistent with a muscarinic M_2 receptor mechanism (Thomas *et al*., 1993; Thomas & Ehlert, 1994). Therefore, the observations in this investigation strongly suggest that acetylcholine released by mATP can also act on muscarinic M_2 receptors to antagonize the relaxant effects of isoproterenol on histamine-induced contraction.

There is some difficulty in accurately measuring the potency of the antagonists used in this study because of the nature of the ileal preparation. Application of mATP results in a transient contractile response, presumably due to a rapid release of acetylcholine followed by its swift removal from the neuroeffector junction. As a consequence of these fast kinetics, acetylcholine and the competitive antagonists do not have time to equilibrate with the receptor, and consequently, the antagonists may behave essentially as irreversible antagonists, even though competitive-like effects are typically observed under experimental conditions favouring equilibrium. This type of quasi-equilibrium has been previously described by Rang (1965). Consistent with this mechanism, the antagonistic profile of the competitive reversible antagonists AF-DX 116 (Figure 4) and NMS (Figures 1 and 3) were similar to that of the irreversible antagonist 4-DAMP mustard (Figure 2). Instead of producing a parallel, rightward shift in the concentration-response curve, AF-DX 116 and NMS produced a decrease in the maximal response to mATP similar to the effects of 4-DAMP mustard.

Presumably, a concentration-dependent relationship exists between mATP and acetylcholine release. If, for instance, acetylcholine release is proportional to mATP concentration over the entire range of the concentration-response curve, the effect of the antagonist on the mATP concentration-response curve should be equivalent to the effect of a muscarinic antagonist on the theoretical acetylcholine concentration-response curve. However, because of the uncertainty in the relationship between mATP and acetylcholine release and the rapid kinetics of acetylcholine release, it is not possible to calculate the K_B of the antagonist precisely. In previous investigations by Czeche *et al*. (1998; 1999), they were able to show good correlation between the pIC_{50} values of antimuscarinic agents obtained from the antagonism of the contractile response elicited to field-stimulation and mATP when compared to each other and to the binding affinities (pA_2 values) of the same antagonists at the muscarinic M_3 receptor. In these investigations, the pIC_{50} values of antagonists (i.e., atropine, p-F-HHSiD, himbacine, and pirenzepine), measured by antagonism of mATP induced contractions in the guinea-pig ileum, were approximately 8 – 16 fold lower then the pA_2 values derived from Schild analysis (Arunlakshana & Schild, 1959).

Nevertheless, if we make the provisional assumption that acetylcholine release is proportional to the concentration of mATP over the range of the concentration-response-curve and that the antagonists AF-DX 116 and NMS behave as irreversible antagonists, it is possible to calculate receptor occupancy caused by AF-DX 116 and NMS using the same method to calculate the receptor inactivation by 4-DAMP mustard (see Methods). Accordingly, we estimate that AF-DX

116 at 1 μM occupies 71% of the receptors mediating the contractile response elicited to mATP following 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol (Figure 4, Table 1). Similar calculations for NMS at 10 nM yielded estimates of 67 and 87% receptor occupancy based on antagonism of the standard contractile response to mATP (Figure 1, Table 1) and that measured after 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol (Figure 3, Table 1), respectively. Knowing receptor occupancy, it is possible to estimate the dissociation constants of the antagonists by rearrangement of the occupancy equation:

$$\text{Occupancy} = [A]/([A] + K_B) \quad (2)$$

where $[A]$ represents the concentration of the antagonist and pK_B represents the dissociation constant of the antagonist for the receptor. We calculated the pK_B values for AF-DX 116 and NMS of 6.39 and 8.82, respectively, for antagonism of the response to mATP after 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol. For the antagonism of the standard contractile response to mATP, the pK_B of NMS was 8.31.

When measured under standard conditions or after 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol, the pK_B values of NMS were approximately the same (i.e., 8.31 and 8.82, respectively). This similarity shows that NMS does not discriminate between the muscarinic subtypes that mediate the response to mATP under the two different conditions. As described above, these muscarinic subtypes are most likely the M_2 (after 4-DAMP mustard-treatment) and the M_3 (standard conditions). However, the estimates of the pK_B values of NMS are approximately one log unit less than the binding affinity values (pK_D) measured in Chinese hamster ovary cells transfected with either the human muscarinic M_2 (9.20) or M_3 (9.53) receptor. These binding estimates were made in a KRB buffer system similar to that used in this study. The discrepancy between binding affinity and functional antagonism might be attributable to a lack of proportionality between the concentration of mATP and the release of acetylcholine. Specifically a cooperative relationship between mATP and acetylcholine release could explain the data. For example, if the concentration of mATP required to elicit a designated level of contraction increases 3 fold in the presence of NMS whereas the concentration of endogenous acetylcholine actually increases 10 fold, then the pK_B value will be under-estimated. This type of error can be corrected by expressing the pK_B value of the antagonist relative to that of a standard, like NMS. This relative measure of antagonism can then be compared with the corresponding relative binding affinity. Using this approach, we estimate that the K_B value of AF-DX 116 is approximately 323.5 fold greater than that of NMS when measured by antagonism of the contractile response to mATP after 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol. Similarly, the dissociation constant of AF-DX 116 measured in binding assays on CHO cells transfected with M_2 receptors was 85 fold greater than that measured for NMS. In contrast, the dissociation constant of AF-DX 116 at cloned M_3 receptors was 2690 fold greater than that of NMS. The better agreement between the relative functional antagonism and relative binding affinity of AF-DX 116 at the M_2 receptor strongly indicates that the M_2 receptor is primarily responsible for mediating the contractile response to endogenous acetylcholine after treatment with 4-DAMP mustard and in the presence of histamine and isoproterenol.

The evidence describe above suggests that there may be a cooperative relationship between the concentration of mATP and the release of acetylcholine. Further evidence for this relationship comes from the steepness of the standard contractile response curve of mATP which exhibited a Hill coefficient of 2.7. In contrast, Hill coefficients of highly efficacious agonists, like acetylcholine, are typically about 2.1 in the standard guinea-pig ileum bioassay (see Ehlert *et al.*, 1999). If there was a proportional relationship between the concentration of mATP and acetylcholine release, then the concentration-response curve of mATP should also exhibit a Hill coefficient of 2.1. The greater observed value of 2.7 suggests that, as the concentration of mATP increases, there is an even greater increase in the release of acetylcholine (i.e., a cooperative relationship between the concentration of mATP and acetylcholine release).

The discrepancy between the observed pK_B value of NMS for antagonizing responses to mATP and the binding affinity of NMS is presumably related to the amount of acetylcholine released and its dependence on the concentration of mATP. Since this discrepancy was similar at both the muscarinic M_2 response to mATP (after 4-DAMP mustard-treatment and in the presence of histamine and isoproterenol) and the muscarinic M_3 response to mATP (standard conditions), then the amount of mATP-induced acetylcholine release in the vicinity of muscarinic M_2 and M_3 receptors is approximately the same in the guinea-pig ileum. This condition implies comparable rates of neurotransmission through both receptor subtypes under physiological conditions.

Collectively, our data are consistent with those of Czeche *et al.* (1998;1999) who postulated that the contractile response to mATP in the guinea-pig ileum is a result of the neurogenic release of acetylcholine acting on muscarinic M_3 receptors. In addition, released acetylcholine can also activate muscarinic M_2 receptors to elicit an indirect contractile response following 4-DAMP mustard-treatment, provided that histamine and isoproterenol are present. Therefore, it seems likely that endogenous acetylcholine acting on muscarinic M_2 receptors may play a physiological role in modulating the relaxant effects of adrenergic stimuli in smooth muscle of the guinea-pig ileum.

This work was supported by National Institutes of Health grant NS 30882. G. Lambrecht thanks the Fonds der Chemischen Industrie (Germany) and the Deutsche Forschuugsgemeinschaft for financial support.

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(Received August 24, 1999
Accepted December 21, 1999)