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Cholinergic facilitation of neurotransmission to the smooth muscle of the guinea-pig prostate gland

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1 Functional experiments have been conducted to assess the effects of acetylcholine and carbachol, and the receptors on which they act to facilitate neurotransmission to the stromal smooth muscle of the prostate gland of the guinea-pig.

2 Acetylcholine and carbachol (0.1 μM–0.1 mM) enhanced contractions evoked by trains of electrical field stimulation (20 pulses of 0.5 ms at 10 Hz every 50 s with a dial setting of 60 V) of nerve terminals within the guinea-pig isolated prostate. In these concentrations they had negligible effects on prostatic smooth muscle tone. The facilitatory effects of acetylcholine, but not those of carbachol, were further enhanced in the presence of physostigmine (10 μM).

3 The facilitatory effects of carbachol were unaffected by the neuropeptide Y Y_1 receptor antagonist BIBP 3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) (0.3 μM, $n=3$) or suramin (100 μM, $n=5$). Prazosin (0.1 μM, $n=5$) and guanethidine (10 μM, $n=5$) alone and in combination ($n=4$), reduced responses to field stimulation and produced rightward shifts of the log concentration-response curves to carbachol.

4 The rank orders of potency of subtype-preferring muscarinic receptor antagonists in inhibiting the facilitatory actions of acetylcholine and carbachol were: pirenzepine > HHSiD (hexahydrosiladifenidol) > *p*F-HHSiD (*para*-fluoro-hexahydrosiladifenidol) ⩾ himbacine, and pirenzepine > HHSiD > himbacine ⩾ *p*F-HHSiD, respectively. These profiles suggest that muscarinic receptors of the M_1-subtype mediate the facilitatory effects of acetylcholine and carbachol on neurotransmission to the smooth muscle of the guinea-pig prostate.

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Abbreviations: BIBP 3226, (*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide; DMPP (1,1-dimethyl-4-phenylpiperazinium iodide; HHSiD, hexahydrosiladifenidol; McN-A-343, (4-hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbanilate chloride; *p*F-HHSiD, *para*-fluoro-hexahydrosiladifenidol

Introduction

The prostate of the guinea-pig, like that of man and in contrast to that of the rat, has a substantial stromal component, comprising a high proportion of smooth muscle cells (Ricciardelli *et al*., 1989). With age, the guinea-pig prostate develops a stromal hyperplasia histologically indistinguishable from that observed in ageing men (Horsfall *et al*., 1994). For these and other reasons including the fact that the guinea-pig prostate exhibits gonadal steroid sensitivity similar to that of the human (Maini *et al*., 1997), the guinea-pig prostate may provide a useful animal model for studies in the human. This possibility is reinforced by the finding that neuromuscular transmission to the smooth muscle of the guinea-pig prostate, as to that in the human prostate, is predominantly sympathetic and noradrenergic in nature (Ohkawa 1983; Haynes & Hill, 1997; Najbar-Kaszkiel *et al*., 1997; Lau *et al*., 1998).

The role(s) of cholinergic nerves within the prostate gland remain uncertain, but participation of acetylcholine in prostatic secretory processes (Farrell & Lyman, 1937; Farnsworth & Lawrence, 1965), and of muscarinic receptors in mitogenic effects (Luthin *et al*., 1997; Rayford *et al*., 1997; McVary *et al*., 1998) have been proposed. The guinea-pig prostate gland, like that in the human (see Dail, 1993 for a review), contains acetylcholinesterase-positive nerve fibres (Lau *et al*., 1998), which densely supplies the stroma as well as the acini of the gland. Acetylcholinesterase-positive staining is, however, not a definitive marker for cholinergic nerves. Moreover, early radioligand receptor autoradiographic studies suggested that muscarinic receptors in the human prostate, while prominent on the secretory epithelium (Lepor & Kuhar, 1984; Hedlund *et al*., 1985; James *et al*., 1989), were present in much lower density in the stroma, consistent with a role of cholinergic nerves in secretory processes. For these reasons, and since muscarinic cholinoceptor agonists have been reported not to be particularly effective in causing contraction of the human prostate (Caine *et al*., 1975; Hedlund *et al*., 1985), the possible role of endogenous acetylcholine in neurotransmission to the prostate stroma has not been extensively further examined. However, it has been proposed by Dail (1993), that cholinergic neurones, probably of sympathetic origin, may also contribute to nerve stimulation-induced contractions of prostatic smooth muscle.

Preliminary experiments in this laboratory (Lau & Pennefather, 1995) indicated that the smooth muscle of the guinea-pig prostate, like that of the human prostate, and in contrast to that of the rat (Lau & Pennefather, 1998), is not particularly responsive to contractile effects of cholinoceptor agonists. However, in our laboratory and in other recent studies, it has been found that atropine reduced field stimulation-induced contractions of the guinea-pig prostate, (Haynes & Hill, 1997; Najbar-Kaszkiel *et al*., 1997; Lau *et al*.,

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1998). In addition, we have observed that anticholinesterases produced a marked atropine-sensitive but hexamethonium-insensitive enhancement of field stimulation-induced contractions of prostate smooth muscle in this species (Lau *et al*., 1998). These observations provide evidence for the proposal that acetylcholine plays a role in neurotransmission to the prostate smooth muscle.

In the present investigation we focus on the role of acetylcholine in neurotransmission to the stromal smooth muscle of the guinea-pig prostate and, in particular, on the muscarinic receptor subtypes mediating the facilitatory effects of muscarinic agonists. There are five subtypes of muscarinic cholinoceptor gene products, four of these correspond to pharmacologically defined receptors (M_1-M_4; see reviews by Eglen *et al*., 1996; Caulfield & Birdsall, 1998). The predominant muscarinic receptor subtype present in the prostate gland shows species variation; for example, M_3 in the rat (Latifpour *et al*., 1991; Yazawa & Honda, 1993, Lau & Pennefather, 1998; Pontari *et al*., 1998) and M_2 in the dog (Fernandez *et al*., 1998). The predominant subtype present in the human prostate is, in contrast, M_1 (Ruggieri *et al*., 1995; Luthin *et al*., 1997). The subtype(s) present in the guinea-pig prostate have not been investigated.

Preliminary accounts of this study have been communicated to The Australasian Society of Clinical and Experimental Pharmacologists and Physiologists (Lau & Pennefather, 1995) and the International Society for Autonomic Neuroscience (Lau *et al*., 1997).

Methods

Animals

Adult male Dunkin-Hartley guinea-pigs (420–700 g) were housed in open runs at 22°C with a 12 h : 12 h light : dark cycle. Rodent chow, fruits, vegetables and water were provided *ad libitum*. Prior approval for animal experimentation was obtained from the Monash University Standing Committee on Ethics in Animal Experimentation (SCEAE Approval No. 95/086 and 95/141).

Organ bath studies

Ventral prostates were removed from guinea-pigs immediately after death by cervical dislocation and exsanguination. As described by us previously (Lau *et al*., 1998), three to four preparations, weighing 51.4±5.1 mg (from a sample of 20 preparations), from each animal were set up under a resting force of 0.5 g in 5- or 10-ml organ baths containing Krebs-Henseleit solution (maintained at 37°C and bubbled with 5% CO_2 in O_2) of the following composition (mM): NaCl, 118.1; KCl, 4.87; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.7; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 2.5. After equilibration for 30 min, the preparations were electrically field stimulated with trains of 20 pulses of 0.5 ms at 10 Hz every 50 s with a dial setting of 60 V (supramaximal) *via* two parallel electrodes (≈0.5 cm apart) incorporated into the tissue holder, and connected to a *Grass S88* stimulator. Isometric contractions were recorded with *Grass FT03C* force-displacement transducers connected to a *MacLab* data acquisition system (Chart 3.3) interfaced with a *Macintosh LC575* computer. The stimulation parameters employed produce tetrodotoxin- and guanethidine-sensitive contractions indicating activation of sympathetic nerve terminals supplying the prostate smooth muscle (Lau *et al*., 1998).

Effects of cholinoceptor agonists

Field stimulated preparations of the guinea-pig prostate were allowed to equilibrate for 30 min before drug addition. Log concentration-response curves to the cholinoceptor agonists, DMPP (1,1-dimethyl-4-phenyl-piperazinium iodide; 1 μM–0.1 mM), carbachol (0.1 μM–1 mM), acetylcholine (0.1 μM–1 mM) and McN-A-343 ((4-hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbanilate chloride; 0.1 μM–0.1 mM) were constructed cumulatively using one log unit concentration increments. Each concentration was added when responses to the previous concentration reached a plateau within 5–10 min after agonist exposure. The effect of nicotine (10 μM) was also investigated.

Experiments were also undertaken to examine the effect of physostigmine (10 μM) on acetylcholine- and carbachol-induced facilitation of field stimulation-induced contractions of the guinea-pig prostatic smooth muscle. The first concentration-response curve to each agonist was constructed in the absence of physostigmine and subsequent curves, at 60 min intervals, were constructed 5–10 min after exposure to physostigmine (10 μM). To determine whether acetylcholine contracted prostatic smooth muscle or enhanced the responses to noradrenaline, discrete log concentration-response curves to noradrenaline were constructed before and 10 min after exposure to acetylcholine (10 μM) in the presence of physostigmine (10 μM). These concentration-response curves to noradrenaline (0.1 μM–1 mM) were constructed with an exposure period of 60 s on a 10 min dose-cycle.

Effects of neurotransmitter antagonists on responses to acetylcholine and/or carbachol

To determine possible sites of action of the cholinoceptor agonists, log concentration-response curves to acetylcholine and/or carbachol were examined upon field stimulated preparations, before and 30 min after exposure to the ganglion blocking agent hexamethonium (0.1 mM), the P_2 purinoceptor antagonist suramin (100 μM), the neuropeptide Y Y_1 receptor antagonist BIBP 3226 (0.3 μM), the α_1-adrenoceptor antagonist prazosin (0.1 μM), and the noradrenergic neurone blocking drug, guanethidine (10 μM).

Effects of subtype-preferring muscarinic receptor antagonists

Log concentration-response curves to acetylcholine in the presence of physostigmine or to carbachol were constructed in the absence and presence of the following subtype-preferring muscarinic receptor antagonists: pirenzepine (M_1; 0.1–1 μM), himbacine (M_2; 1 μM), HHSiD, (M_1/M_3; 0.1–1 μM) or *p*F-HHSiD, (M_3; 1 μM), with an antagonist incubation period of 30 min, and in a subset of experiments with pirenzepine (1 μM), an incubation period of 90 min. Only one antagonist concentration was tested in any one tissue preparation. Control experiments were conducted in parallel to correct for any tissue sensitivity changes due to time and/or vehicle (bath concentrations of up to 0.01% ethanol).

Measurement and analysis of data

The effects of agonists on the magnitude of responses to electrical field stimulation were expressed as percentage

increases of mean basal field stimulation-induced responses. The mean peak force developed (in g) of four stimulation-induced responses was determined just prior to the initial agonist addition to estimate mean basal field stimulation-induced force. The corresponding estimates of mean peak force developed when the response to each concentration had reached a plateau (5–10 min after each dosage increment) were determined in the absence and presence of antagonists. Log concentration-response curves to acetylcholine and carbachol before and after exposure to antagonists and/or physostigmine were constructed by pooling data from individual curves.

The effects of acetylcholine on noradrenaline-induced contractions of the guinea-pig prostatic smooth muscle were determined by measuring the magnitude of contractile force developed (in g) in response to each concentration of noradrenaline in the absence and presence of acetylcholine.

Non-linear regression analyses of log concentration-response curves were undertaken using the *GraphPad PRISM* software program. To estimate the potencies of physostigmine in enhancing the effects of acetylcholine and of antagonists in inhibiting the effects of acetylcholine and carbachol, the slopes of the mean log concentration-response curves to the agonists in the absence and presence of these drugs were compared. When the slopes did not differ significantly, as indicated by overlap of the 95% confidence limits of these slopes, concentration ratios were determined. Mean estimates of apparent dissociation constants (K_B) of the muscarinic receptor subtype-preferring antagonists, expressed as apparent pK_B, for each concentration used were calculated using the equation: apparent K_B = (antagonist concentration)/(concentration ratio -1) (Furchgott, 1972).

Data are presented as mean value ± standard error of the mean (s.e.mean); n represents the number of experimental animals. Statistical evaluation of data was performed using one and two-way repeated measures analyses of variance (ANOVA), Student's paired or unpaired t-tests, where appropriate. In all cases, values of $P<0.05$ were considered significant.

Drugs

The following drugs were used: (−) arterenol (noradrenaline) bitartrate, acetylcholine chloride, carbachol (carbamylcholine chloride), 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), physostigmine hemisulphate, prazosin (Sigma), guanethidine (Ciba-Geigy), hexamethonium tartrate (May & Baker), hexahydrosiladifenidol (HHSiD) hydrochloride, *para*-fluoro-hexahydrosiladifenidol (*p*F-HHSiD) hydrochloride, 4-hydroxy-2-butynyl-1-trimethylammonium-*m*-chlorocarbamilate chloride (McN-A-343) (Research Biochemicals International) and nicotine hydrogen (+)-tartrate (BDH). Pirenzepine dihydrochloride and himbacine hydrochloride were gifts from Dr K. Thomae, Boehringer Ingelheim and Prof W.C. Taylor of University of Sydney, Australia, respectively. BIBP 3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) was a gift from Dr Margaret Morris, University of Melbourne. Suramin was a gift from Bayer, Australia.

Noradrenaline was dissolved and diluted in a catecholamine diluent (mM): NaCl, 154.0; NaH_2PO_4, 1.2; ascorbic acid, 0.2. All other drugs except HHSiD and *p*F-HHSiD were dissolved in distilled water. Stock concentrations (1 mM) of HHSiD and *p*F-HHSiD were prepared in absolute ethanol. Dilutions to working concentrations were made in distilled water.

Results

Effects of agonists on prostatic smooth muscle tone

Cumulative addition of acetylcholine and carbachol (0.1 μM–0.1 mM), did not cause any appreciable effect on the guinea-pig prostatic smooth muscle tone. In some preparations, at higher concentrations (> 0.1 mM), they produced small increases in the smooth muscle tone. Nicotine (10 μM), DMPP (1 μM–0.1 mM) and McN-A-343 (0.1 μM–0.1 mM) were without effect on the prostatic smooth muscle tone. Noradrenaline (0.1 μM–1 mM) produced concentration-dependent contractions of the guinea-pig prostatic smooth muscle; its effects were not modified in the presence of acetylcholine (10 μM) and physostigmine (10 μM) (Figure 1).

Effects of agonists on contractile responses evoked by field stimulation

As reported previously (Lau *et al*., 1998), repetitive trains of stimuli (20 pulses of 0.5 ms at 10 Hz every 50 s) evoked reproducible contractions of the guinea-pig prostatic smooth muscle; the magnitudes (0.12 ± 0.02 g; sample $n=12$) of these responses were consistent over the experimental period. Application of acetylcholine and carbachol caused concentration-related enhancement of the field stimulation-induced contractions (Figure 2). These effects were reversible after several washouts. As shown in Figure 3, in the absence of physostigmine, acetylcholine was less potent than carbachol in enhancing field stimulation-induced contractions. Control experiments indicated that the first and second log concentration-response curves to carbachol, constructed in the absence and presence of the ethanol vehicle at intervals of 60 min, were not significantly different ($P > 0.05$, two-way ANOVA; Figure 4), but there was a small rightward shift in the position of the third curve. The magnitudes of the basal field stimulation-induced contraction and the increase induced by the highest concentration (0.1 mM) of carbachol applied were similar in each of these curves.

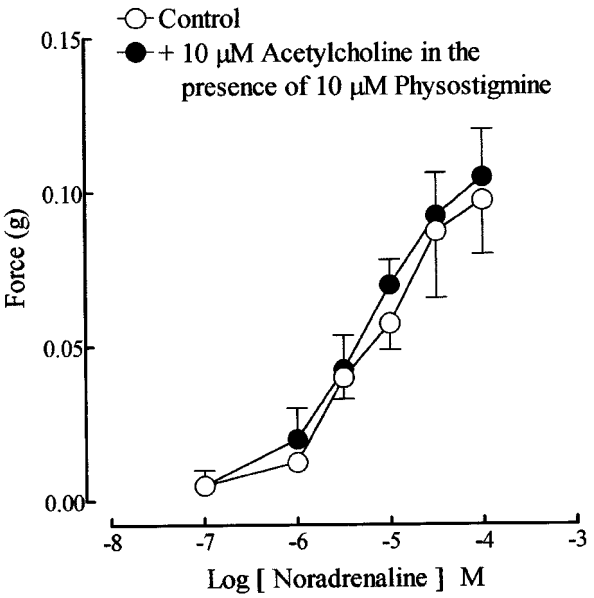

Figure 1 Mean log sequential concentration-response curves to noradrenaline on the guinea-pig prostatic preparations before and after exposure to acetylcholine (10 μM) in the presence of physostigmine (10 μM). Data are shown as mean values with vertical lines representing s.e.mean from four experiments.

As reported previously (Lau *et al*., 1998), physostigmine (10 μM) potentiated the stimulation-induced contractions of the guinea-pig prostate. In the present experiments it produced a 66.7 $\pm$ 7% (n = 25) increase in the magnitude of the responses. In time control experiments, physostigmine-induced effects were maintained throughout the 20–25 min period needed for construction of concentration-response curves to acetylcholine. In its presence, the second mean log concentration-response curves to acetylcholine were shifted to the left in a parallel manner with a potency ratio of 14.8 (95% confidence limits: 11.9, 18.4; d.f. = 62; n = 8) (Figure 5). The position of the

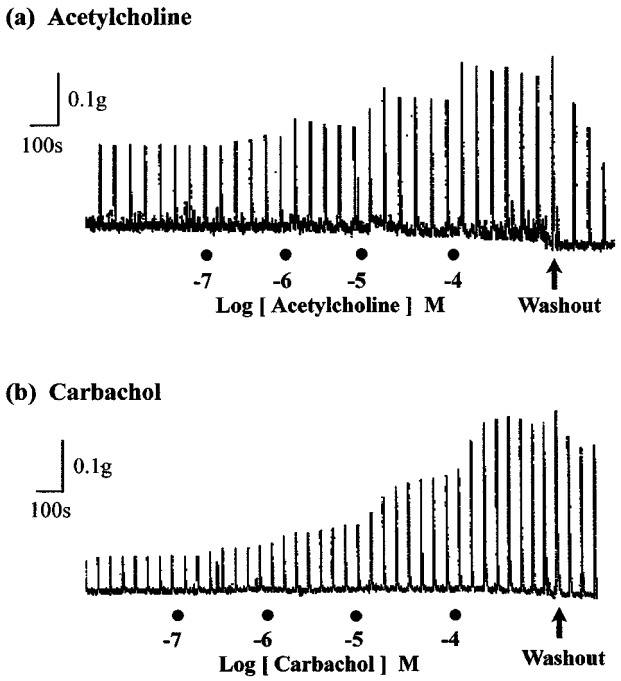

Figure 2 Representative traces showing the enhancing effect of increasing concentrations of (a) acetylcholine and (b) carbachol on field stimulation (trains of 20 pulses of 0.5 ms at 10 Hz every 50 s, 60 V)-induced contractions of the guinea-pig prostatic smooth muscle preparations.

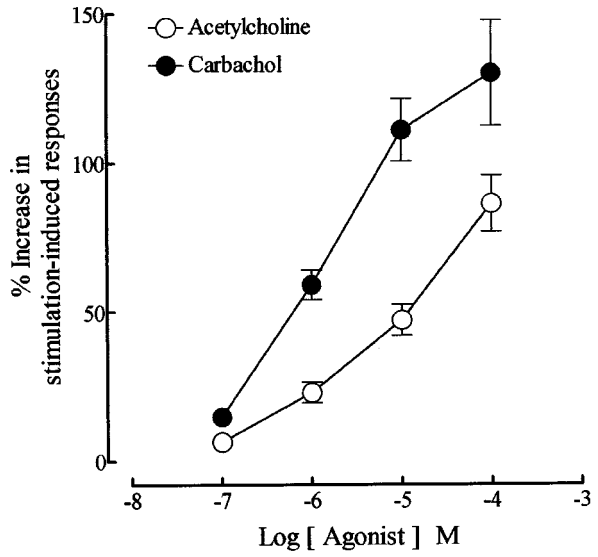

Figure 3 Mean log concentration-response curves for the enhancing effect of acetylcholine (in the absence of physostigmine) and carbachol on field stimulation (trains of 20 pulses of 0.5 ms at 10 Hz every 50 s, 60 V)-induced contractions of the guinea-pig prostatic preparations. Data are shown as mean values with vertical lines representing s.e.mean from 16 experiments.

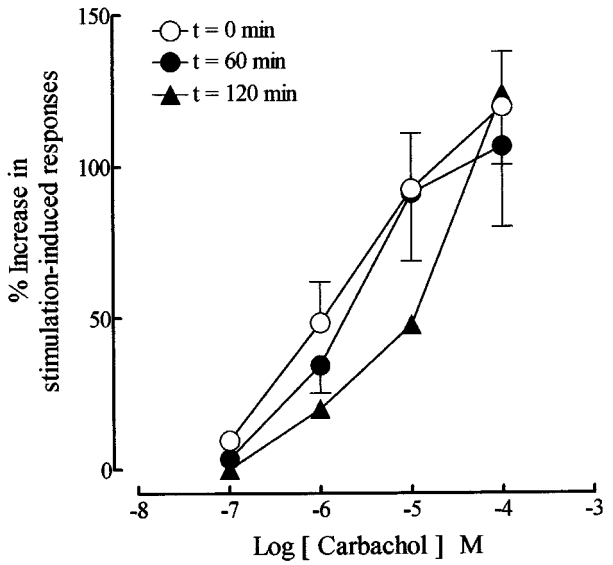

Figure 4 Mean log concentration-response curves to carbachol at t = 0 min (in the absence of ethanol), t = 60 min (in the presence of 0.01% ethanol) and t = 120 min (in the presence of 0.01% ethanol) on field stimulation (trains of 20 pulses of 0.5 ms at 10 Hz every 50 s, 60 V)-induced contractions of the guinea-pig prostatic preparations. Data are shown as mean values with vertical lines representing s.e.mean from 4–5 experiments.

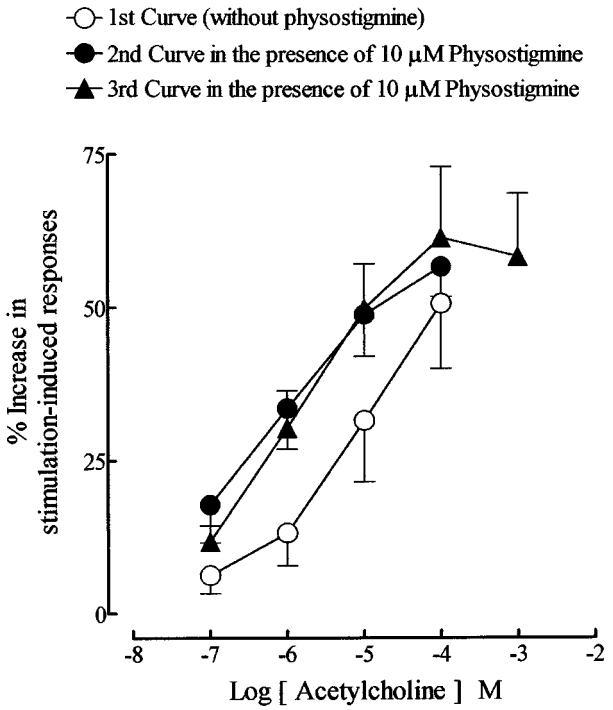

Figure 5 The effects of physostigmine (10 μM) on the mean log concentration-response curves for the enhancing effects of acetylcholine on field stimulation (trains of 20 pulses of 0.5 ms at 10 Hz every 50 s, 60 V)-induced contractions of the guinea-pig prostatic preparations. The first curve to acetylcholine was constructed in the absence of physostigmine. Physostigmine was added 5–10 min before construction of the second and third curves to acetylcholine. Data are shown as mean values with vertical lines representing s.e.mean from eight experiments.

third curve to acetylcholine, constructed in the presence of physostigmine, was not significantly different from the second curve ($P > 0.05$, two-way ANOVA) (Figure 5). The enhancement of field stimulation-induced contractions induced by the highest concentration (0.1 mM) of acetylcholine applied was not significantly altered by physostigmine. Physostigmine was without effect on carbachol-induced facilitation ($n = 5$; $P > 0.05$, two-way ANOVA).

DMPP (1 μM – 0.1 mM, $n = 3$), nicotine (10 μM, $n = 4$) and McN-A-343 (0.1 μM – 0.1 mM, $n = 3$) were without significant effect on the field stimulation-induced contractions ($P > 0.05$, one-way ANOVA) of the prostatic smooth muscle.

Effects of antagonists on responses to acetylcholine and/or carbachol

Neither hexamethonium (0.1 mM) nor the neuropeptide Y Y_1 receptor antagonist BIBP 3226 (0.3 μM) modified the magnitude of stimulation-induced contractions or the facilitatory effects of acetylcholine and/or carbachol on stimulation-induced contractions of the guinea-pig prostatic smooth muscle ($n = 3-4$; $P > 0.05$, two-way ANOVA). Exposure of the prostatic preparations to suramin (100 μM, $n = 5$), prazosin (0.1 μM, $n = 5$), guanethidine (10 μM, $n = 5$) and prazosin and guanethidine in combination ($n = 4$) caused $20 \pm 12\%$, $73 \pm 7\%$, $82 \pm 4\%$ and $86 \pm 6\%$ inhibition respectively, of the magnitude of stimulation-induced contractions. The facilitatory effects of carbachol were unaltered in the presence of suramin. Prazosin and guanethidine, alone and in combination produced small but significant decreases to the carbachol-induced responses ($P < 0.05$, two-way ANOVA). Log concentration-response curves to carbachol were shifted 2.52-(95% confidence limits: 2.43, 2.60; d.f. = 58), 3.92-(95% confidence limits: 3.26, 4.73; d.f. = 61) and 3.66-(95% confidence limits: 3.62, 3.71; d.f. = 48) fold to the right in the presence of prazosin, guanethidine, alone and in combination, respectively.

Effects of subtype-preferring muscarinic receptor antagonists

All the subtype-preferring muscarinic receptor antagonists used (pirenzepine 0.1 – 1 μM, HHSiD 0.1 – 1 μM and *p*F-HHSiD 1 μM) except himbacine (1 μM) caused slight but significant inhibition of the magnitude of stimulation-induced contractions of the guinea-pig prostatic smooth muscle preparations ($P < 0.05$, Student's paired *t*-tests). The maximum extent of inhibition, of $33 \pm 11\%$ ($n = 5$) was seen with HHSiD (0.1 μM).

In the presence of these subtype-preferring antagonists, log concentration-response curves to acetylcholine in the presence of physostigmine and to carbachol were shifted to the right without significant suppression of the responses to maximal concentrations of agonist applied. Estimates of the affinities/potencies (apparent pK_B values) of these antagonists in inhibiting responses to the agonists are shown in Table 1. The effect of extension of the incubation period for pirenzepine from 30 to 90 min was examined; there was no significant change in the apparent pK_B values ($n = 5$; $P > 0.05$, Student's unpaired *t*-tests).

Correlation analysis between the apparent pK_B values of antagonists in inhibiting the facilitatory effects of acetylcholine and carbachol on stimulation-mediated contractions of the guinea-pig prostate and published mean estimates of their affinities (pA_2 or apparent pK_B values) at muscarinic receptor systems showed a significant correlation with affinity for the muscarinic M_1 receptor using carbachol as the agonist. Estimates of r^2 and associated P values are listed in Table 2.

Discussion

The aims of this study were: (1) to examine the effects of cholinoceptor agonists on smooth muscle tone and on neuromuscular transmission to the guinea-pig isolated prostate gland; and (2) to determine the muscarinic receptor subtype mediating the effects of agonists on the prostate of this species.

Acetylcholine and carbachol produced little or no direct effect on the smooth muscle tone of the guinea-pig isolated prostate. Cohen & Drey (1989) have previously reported that, in contrast to histamine and α-adrenoceptor agonists, carbachol produced minimal contractile activity in the prostatic smooth muscle preparations of the guinea-pig. Similarly, in the human prostate, although acetylcholine contracts the prostatic capsule it has minimal effect on the prostate stroma (Caine *et al*., 1975; Hedlund *et al*., 1985). Species variations in the responsiveness of the prostate to cholinoceptor agonists do occur as the contractile effect of carbachol is prominent in rat (Lau & Pennefather, 1998) and dog prostates (Fernandez *et al*., 1998).

Despite a lack of direct contractile effect, acetylcholine and carbachol both enhanced neurotransmission to the guinea-pig prostate gland in a concentration-dependent manner. Carbachol was the more potent. In the presence of the anticholinesterase, physostigmine, the facilitatory effects of acetylcholine, but not of carbachol, were significantly enhanced, confirming the presence of cholinesterase in the prostate from the guinea-pig. Since the effects of acetylcholine and carbachol were unaltered by the ganglion blocking agent, hexamethonium, and since DMPP and nicotine, were without effect on field stimulation-induced contractions of the prostatic smooth muscle, it can be assumed that nicotinic receptors are not involved in the responses to acetylcholine and carbachol,

Table 1 Apparent pK_B values for muscarinic antagonists *versus* acetylcholine and carbachol on field stimulated preparations of the guinea-pig prostate

Antagonist (μM)	*Apparent* pK_B *values*	
	Acetylcholine[a]	*Carbachol*
Pirenzepine (0.1 – 1 μM)	7.64 ± 0.14	7.45 ± 0.15
Himbacine (1 μM)	6.78 ± 0.14	6.68 ± 0.33
HHSiD (0.1 – 1 μM)	7.34 ± 0.21	7.23 ± 0.44
*p*F-HHSiD (1 μM)	6.91 ± 0.26	6.39 ± 0.27

Results are expressed as mean apparent pK_B values ± s.e.-mean from 4 – 6 experiments. [a]In the presence of physostigmine (10 μM).

Table 2 Coefficients of determination (Pearson's r^2) between prostate apparent pK_B and literature values at muscarinic receptor subtypes

Muscarinic receptor subtypes	r^2 versus *Acetylcholine*	r^2 versus *Carbachol*
M_1	0.86[a]	0.97[b]
M_2	0.42	0.12
M_3	0.13	0.12
M_4	0.60	0.20
M_5	0.00	0.06

Affinity estimates at functional muscarinic receptor subtypes were obtained from Lambrecht *et al*. (1989), Eglen *et al*. (1990) and Doods *et al*. (1993). [a]$P < 0.075$; [b]$P < 0.014$.

despite the possibility that ganglion cells may be located in close proximity to the prostate in this species (Lamano Carvalho *et al*., 1986). Thus the facilitatory effects of acetylcholine and carbachol, and those of the anticholinesterases examined in our previous study (Lau *et al*., 1998), are likely to be mediated by a muscarinic receptor.

The mechanism/s through which acetylcholine and carbachol act to facilitate neurotransmission to the prostate were examined indirectly. Noradrenaline produces contractions of guinea-pig prostate smooth muscle by activation of α_{1L}-adrenoceptors (Pennefather *et al*., 1999). Acetylcholine, in the presence of physostigmine, did not enhance these contractions. This finding is in contrast to that reported for the guinea-pig vas deferens (Sjostrand, 1973; Sjostrand & Swedin, 1974), indicating an absence of synergism between the two neurotransmitters at postjunctional receptors in the prostate gland. Our findings that neither suramin nor the neuropeptide Y Y_1 receptor antagonist BIBP 3226 (Rudolf *et al*., 1994), affected responses to carbachol indicated lack of an effect on the release or actions of either ATP or neuropeptide Y. Previous reports by Cohen & Drey (1989) indicated that neuropeptide Y was without effect on guinea-pig prostate smooth muscle. Similarly, although suramin caused some decrease in the response to field stimulation in the guinea-pig prostate in our present and previous (Lau *et al*., 1998) studies; surprisingly, ATP was without contractile effect on this tissue (Lau, 1998). Taken together, these observations raise the possibility that the cholinoceptor agonists may produce facilitation of neurotransmission to the guinea-pig prostate by enhancing noradrenaline overflow, but this possibility needs further examination.

Operational characterization of the four pharmacologically defined muscarinic receptor subtypes (M_1-M_4) is commonly achieved by obtaining potency profiles of a series of subtype-preferring muscarinic receptor antagonists (reviews by Caulfield, 1993; Eglen *et al*., 1996; Caulfield & Birdsall, 1998). Pirenzepine (M_1), himbacine (M_2/M_4), HHSiD (M_1/M_3) and *p*F-HHSiD (M_3) were employed in the present study. We have previously reported that atropine (1 μM) partially reduced field stimulation-induced contractions of guinea-pig prostate stroma (Lau *et al*., 1998). With the exception of himbacine, the subtype-preferring antagonists we used in this study also partially reduced responses to field stimulation. The effects of the antagonists were not established with sufficiently low concentrations to allow estimation of the relative potencies as a possible guide to the nature of the receptor subtype/s involved, as our major aim in this study was to determine the subtype of muscarinic receptors mediating the facilitatory effects of exogenously administered acetylcholine and carbachol on neurally-mediated contractions.

The potencies of the muscarinic receptor subtype-preferring antagonists in inhibiting the facilitatory effects of the choline esters are broadly consistent with agonist actions at muscarinic M_1 receptors. The potency rank orders (with apparent pK_B values in parenthesis) *versus* acetylcholine, namely pirenzepine (7.64) > HHSiD (7.34) > *p*F-HHSiD (6.91) $\geqslant$ himbacine (6.78) and *versus* carbachol, namely pirenzepine (7.45) > HHSiD (7.23) > himbacine (6.68) $\geqslant$ pF-HHSiD (6.39) were similar except for the order of himbacine and *P*F-HHSiD.

The pK_B estimates of pirenzepine (7.45–7.64) against both agonists are slightly lower than literature values obtained at other M_1 systems (8.1–8.5, from reviews by Hulme *et al*., 1990; Caulfield, 1993; Eglen & Watson, 1996), but are too high to indicate actions at muscarinic M_2 and M_3 receptors (6.8 and 6.9, respectively from Table 2, Eglen & Watson, 1996). They are comparable with those reported for interactions with muscarinic M_4 receptors (7.7–8.1, Caulfield, 1993). However, the relatively low pK_B values (6.68–6.78) obtained for the muscarinic M_2/M_4-receptor preferring antagonist, himbacine, suggest that neither muscarinic M_2 nor M_4 receptors are important in mediating the facilitatory effects of acetylcholine and carbachol.

HHSiD ($M_1 = M_3 > M_2$) has a relatively high affinity for muscarinic M_1 (pA_2 value of 7.9 in rabbit vas deferens) and for M_3 (pA_2 value of 8.0 in guinea-pig ileum) receptors but a low potency at muscarinic M_2 receptors (pA_2 value of 6.5 in rat atria) (Lambrecht *et al*., 1989). Therefore it is a useful tool to distinguish muscarinic M_1 or M_3 receptors from M_2 receptors. The relatively high pK_B estimates obtained for HHSiD (7.23–7.34) *versus* acetylcholine and carbachol in facilitating neurotransmission to the prostate further substantiate the non-involvement of muscarinic M_2 receptors in this effect. These estimates for this antagonist, are however, lower than those observed by Lambrecht *et al*. (1989) in M_1 and M_3 systems. It is unclear why lower estimates for HHSiD and for pirenzepine were obtained in the present study. It is unlikely that the incubation period is too short since other studies have used similar incubation periods, (Doods *et al*., 1993; Roffel *et al*., 1993; Kerr *et al*., 1995). Indeed extension of the incubation period for pirenzepine *versus* carbachol to 90 min was without effect on the potency estimate.

*p*F-HHSiD ($M_3 > M_1 > M_2$) was included to differentiate between muscarinic M_1 and M_3 receptors because of its relatively greater affinity for the latter subtype. At muscarinic M_1, M_2 and M_3 receptors, it exhibits pA_2 values of 7.2–7.5, 6.0–6.9 and 7.8–7.9, respectively (Caulfield, 1993). The relatively low pK_B estimates obtained for *p*F-HHSiD (6.39–6.91) in the present study clearly do not support the view that the neuromodulatory effect of the agonists is mediated by activation of muscarinic M_3 receptors.

Taken together, the antagonist potency profiles obtained in this study are compatible with the possibility that the cholinoceptor agonists facilitate neurotransmission to the guinea-pig prostatic smooth muscle mainly *via* muscarinic M_1 receptors. As shown in Table 2, the potencies of the subtype-preferring antagonists *versus* carbachol-induced facilitation of neurotransmission in the present study correlate well with those reported in the literature for muscarinic M_1 receptors. In contrast, poor correlation with literature values reported for the other muscarinic receptor subtypes was observed. While muscarinic receptor heterogeneity cannot be excluded, there were only small (less than 5 fold) disparities between the present estimates for HHSID and pirenzepine and published values for the M_1 receptor.

There is evidence for the presence of both facilitatory and inhibitory prejunctional muscarinic receptors of the M_1 subtype on both sympathetic and/or parasympathetic neurones supplying other genito-urinary organs (Eltze, 1988; Somogyi & de Groat, 1990; Somogyi *et al*., 1994; 1996; 1997). In our study the putatively M_1 receptor-selective agonist McN-A-343 was without effect on neurotransmission to the guinea-pig prostate smooth muscle. While this finding would suggest that M_1 receptors may not mediate the effects of cholinoceptor agonists it should be noted that McN-A-343 is, however, a partial agonist at muscarinic M_1-M_4 receptors (Lazareno *et al*., 1993), and this may explain its lack of effect. Moreover its effects on neurotransmission to the male genito-urinary tract are species-related. Thus while it inhibits neurotransmission to the rabbit vas deferens by activation of a prejunctional M_1 receptor (Eltze *et al*., 1988), it enhances that to the guinea-pig vas deferens by activation of an M_2 receptor (Walsh *et al*., 1995).

In conclusion, this study establishes for the first time that acetylcholine and carbachol can facilitate neurotransmission to the smooth muscle of the prostate from the guinea-pig. While it is unknown whether similar facilitation occurs with the human prostate, it is of interest that muscarinic M_1 receptors are likely to be involved in mediating these actions in the guinea-pig prostate. This is of particular interest since radioligand binding and immunocytochemical studies by Ruggieri *et al*. (1995) have indicated a preponderance of muscarinic receptors of the M_1 subtype in the human prostate. Thus guinea-pig and human prostates differ from those in dog and rat firstly in that they do not contract in response to choline esters and secondly in that M_1 receptors are prominent only in prostates from the former two species.

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