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Potentiation by cholinoceptor agonists of contractions to field stimulation of rat vas deferens

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1 Cholinoceptor agonists (arecoline ≃ carbachol > acetylcholine > pilocarpine) potentiated contractions to field stimulation of rat vas deferens via the activation of an atropine-sensitive muscarinic receptor.

2 The potentiating effect of carbachol was dependent on the level of calcium in the medium, being more potent at higher calcium concentrations.

3 The potentiating effect of carbachol was more pronounced in the epididymal than in the prostatic segment but was not attenuated by prazosin, an α_1-adrenoceptor antagonist.

4 Carbachol did not significantly modify the direct contractile effects of noradrenaline nor alter the field-stimulation-evoked release of noradrenaline from the epididymal vas deferens.

5 It is concluded that the potentiating effect of cholinoceptor agonists on the contractions to field stimulation in the rat vas deferens was not a result of an enhancement of adrenergic neurotransmission.

Introduction

It is well documented that acetylcholine can enhance the contractile response to nerve stimulation in both guinea-pig (Sjöstrand, 1973) and rat (Graham *et al.*, 1968; Liao & Freer, 1983) vas deferens. The molecular mechanism underlying this action, however, remains elusive. In preliminary experiments it was found that carbachol, like acetylcholine, can potentiate the muscle contractions to field stimulation in the rat vas deferens (Figure 1), and this effect was more pronounced in the epididymal than in the prostatic segment (Figure 2). Since neurogenic contractions in the rat vas deferens have been shown to comprise two components with an α-adrenergic component being predominant in the epididymal portion and a 'non-adrenergic' component being predominant in the prostatic segment (McGrath, 1978; Brown *et al.*, 1983), the present study was undertaken to evaluate the pharmacological characteristics of this cholinergic action as well as to examine whether carbachol potentiated the contractions to field stimulation via an enhancement of adrenergic transmission. Preliminary accounts of these observations were presented at the IUPHAR 9th International Congress of Pharmacology (Lee, 1984).

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Methods

Tissue preparation for bioassay

Adult male Sprague-Dawley rats weighing 250–300 g were used. The animals were killed by cervical dislocation. Vasa deferentia were removed and bisected into prostatic and epididymal halves. Each tissue was suspended in a 5 ml water-jacketed organ bath containing oxygenated (O_2:CO_2, 95:5%) Krebs-bicarbonate solution (composition in mM: NaCl 127, KCl 2.5, $CaCl_2$ 1.8 or as otherwise stated in text, $MgSO_4$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 10). The bath was maintained at 37°C.

Field stimulation of the isolated vas deferens

After an equilibration period of 30 min, the vas deferens was stimulated transmurally by means of a Grass stimulator with two platinum ring electrodes at supramaximal voltage (60–80V; 0.1 Hz, 1 ms or as otherwise stated in text), and the contractions were recorded isometrically on a Beckman R511A polygraph under a resting load of 1 g using a Gould UC-3 force-displacement transducer. Dose-response curves of various drugs were constructed in a cumulative fashion. Stepwise increases in drug con-

centrations were made at 3 min intervals or after the response had stabilized. Affinity of antagonist was evaluated by measuring the pA_2 value (− log of the concentration of antagonist reducing the effect of a double dose of agonist to that of a single dose) according to the method of Arunlakshana & Schild (1959).

Direct spasmogenic test on the isolated vas deferens

The contractions were recorded isometrically under a resting load of 1 g. After an equilibration period of 30 min, dose-response curves of various drugs were determined in a non-cumulative fashion. Drugs were applied at 5 min intervals and allowed a contact time of 1–2 min or until the response had reached its peak. The EC_{50} values (concentrations of drugs required to give a half maximal response) of the drugs were determined from their respective dose-response curves.

Noradrenaline release from the isolated epididymal vas deferens

To determine the release of [^{3}H]-noradrenaline, the epididymal vas deferens was mounted in a 5 ml organ bath as described in the previous section. The oxygenated Krebs-bicarbonate solution contained Na_2EDTA 10 $\mu g\ ml^{-1}$ and ascorbic acid 20 $\mu g\ ml^{-1}$ to prevent oxidative destruction of catecholamines.

After preincubation with [^{3}H]-noradrenaline 5×10^{-7} M for 10 min at 37°C, the tissue was washed with 10 changes of Krebs-bicarbonate solution at 3 min intervals. The tissue was then stimulated at supramaximal voltage (0.1 Hz, 1 ms) for 10 min in the absence or presence of cholinoceptor agonists. At the end of the experiment, the tissue was collected and solubilized in 2 ml of NCS tissue solubilizer (Amersham) overnight. The radioactivities in the tissue and in the medium were determined by liquid scintillation counting.

The release of endogenous noradrenaline was measured essentially according to the method of Oishi *et al.* (1983). In brief, after 30 min equilibration in Krebs-bicarbonate solution containing Na_2EDTA 10 $\mu g\ ml^{-1}$ and ascorbic acid 20 $\mu g\ ml^{-1}$, the tissue was electrically stimulated (70 V, 2.5 Hz, 0.5 ms) for 1 min in the absence or presence of carbachol 4 μM. Ten min after stimulation the tissue was collected and homogenized in 1 ml 0.4 M ice-cold perchloric acid. The amount of noradrenaline in the tissue homogenate and in the incubation medium were determined by electrochemical detection coupled with high performance liquid chromatography (Zaczek & Coyle 1982).

Release results are expressed as fractional rate constants (defined as the percentage of total tissue content released per minute).

Drugs and sources

[^{3}H]-noradrenaline (8.0 Ci $mmol^{-1}$, Amersham); prazosin HCl (gift, Pfizer); atropine sulphate, arecoline HBr, pilocarpine HCl, carbachol HCl, acetylcholine HCl and (−)-noradrenaline bitartrate (Sigma) were used.

Statistical analysis

The results, unless otherwise stated, are expressed as the mean ± s.e.mean. Data, where appropriate, were compared using Student's paired t test and were considered to be significantly different when $P < 0.05$.

Results

Specificity of cholinergic potentiation

Carbachol (10^{-7}–10^{-4} M) caused a dose-dependent and stable potentiation of the muscle contractions to field stimulation in the rat vas deferens (Figure 1). Significant potentiation was observed at 3×10^{-7} M and reached a maximum at approximately 1×10^{-4} M. In contrast to its marked potentiating effect on the stimulated contractile response, carbachol had little or no effect on the resting tension (Figure 1) and displayed minimal direct contractile effect on the unstimulated vas deferens (data not shown). Carbachol gave a more pronounced enhancement of contractile responses in the epididymal segment with the magnitude of maximal potentiation being 3 times higher than that obtained in the prostatic segment (Figure 2). The EC_{50} for carbachol, however, did not

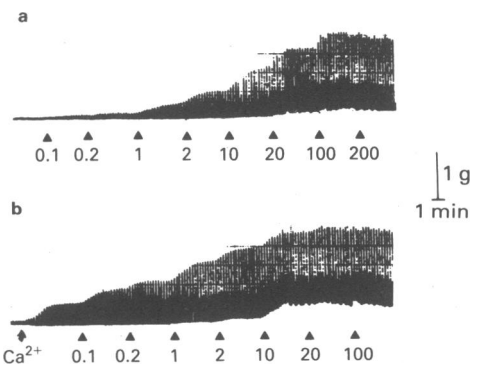

Figure 1 Potentiating effect of carbachol on contractions to field stimulation in the epididymal rat vas deferens (0.1 Hz, 1 ms, supramaximal voltage) at 2 different calcium levels: (a): 1.8 mM Ca^{2+}; (b): 5.4 mM Ca^{2+}. Drugs were added at (▲). Numerals indicate the concentrations of carbachol in μM.

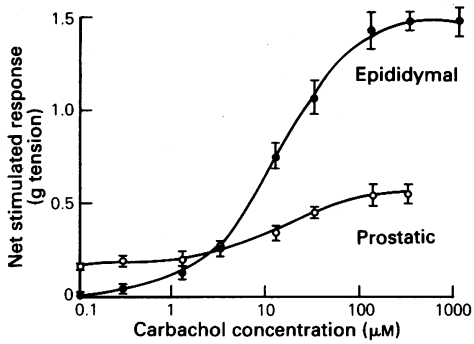

Figure 2 A comparison of the potentiating effects of carbachol on contractions to field stimulation in the epididymal (●) versus the prostatic (○) segments of the rat vas deferens. The results are the mean ± s.e.mean (indicated by vertical bars) of 5 separate experiments.

differ significantly between the epididymal (12.5 ± 2.2 μM) and the prostatic (16.0 ± 3.1 μM) segments.

Other cholinoceptor agonists including arecoline, acetylcholine and pilocarpine potentiated the contractile response in the epididymal vas deferens with EC_{50}s of 9.5, 56 and 85 μM respectively (Figure 3). Arecoline, acetylcholine and carbachol gave similar maxima of potentiation. Pilocarpine, on the other hand, appeared to be a partial agonist and gave a maximal potentiation that was only 1/3 that of the other agonists tested. This observation was reminiscent of the low efficacy of pilocarpine reported for other cholingeric systems (Furchgott & Bursztyn, 1967).

Atropine (2–50 nM) did not significantly modify the basal contractions to field stimulation in the epididymal vas deferens but it caused a parallel shift of

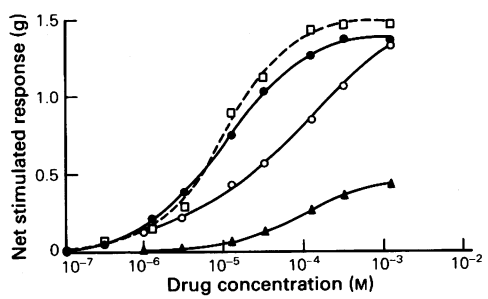

Figure 3 Potentiation of contractions to field stimulation in epididymal rat vas deferens by cholinoceptor agonists: arecoline (□), carbachol (●), acetylcholine (○) and pilocarpine (▲). Data represent the mean of 3 separate experiments which varied by less than 20%.

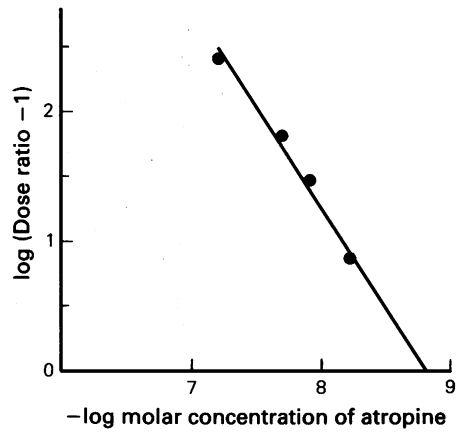

Figure 4 Arunlaksahana & Schild (1959) plot to estimate the potency of atropine as an antagonist of carbachol's potentiating effect on contractions to field stimulation in rat vas deferens. Data shown are those of a typical experiment. $pA_2 = 8.7$.

the dose-response curves for carbachol potentiation to the right in a dose-dependent manner. When analysed with an Arunlakshana & Schild plot, the pA_2 value of atropine was estimated to be 8.69 ± 0.07 ($n = 7$, Figure 4). Atropine (20 nM) antagonized the potentiating effects of acetylcholine and arecoline, but did not modify the potentiation caused by eledoisin and its related tachykinins (data not shown).

Effect of calcium on the potentiating action of carbachol

Carbachol was more potent in potentiating the contractions to field stimulation at higher calcium concentrations. The EC_{50} of carbachol at 5.4 mM calcium (0.4 μM) was about 160 times lower than that at 0.9 mM calcium (65 μM). The magnitude of the maximal stimulated tension caused by carbachol was also sensitive to the external calcium level, being 0.28, 0.7, 1.5 and 1.02 g tension at 0.9, 1.44, 1.8 and 5.4 mM calcium respectively (Figure 5). The apparently smaller effect of carbachol on the stimulated response observed at 5.4 mM calcium presumably reflected the non-additive nature of the effects of calcium and carbachol. As illustrated in Figure 1, the tension of the electrically-evoked contractions was increased by increasing the calcium level in the medium and reached a maximum of 0.4 ± 0.02 g tension ($n = 5$) at 5.4 mM calcium. Since the maximal contractile responses to carbachol obtained at 1.8 mM and 5.4 mM calcium did not differ significantly from each other (Figure 1) the net stimulated response was therefore lower at 5.4 mM calcium.

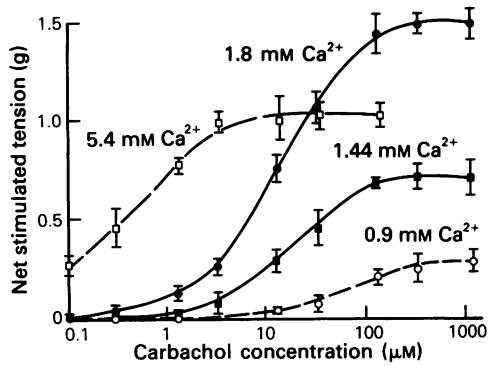

Figure 5 The influence of external calcium concentration on the potentiating effect of carbachol on the evoked response of the epididymal rat vas deferens to field stimulation (0.1 Hz, 1 ms, supramaximal voltage): Ca^{2+} 5.4 mM (□); 1.8 mM (●); 1.44 mM (■); 0.9 mM (○). Each point is the mean ± s.e.mean (indicated by vertical bars) of 3 separate experiments.

Effect of carbachol on adrenergic neurotransmission

Neither carbachol (4 μM) nor acetylcholine (10 μM) significantly modified the field stimulation-evoked release of [^{3}H]-noradrenaline (70V, 0.1 Hz, 1 ms, 10 min) or endogenous noradrenaline (70 V, 2.5 Hz, 0.5 ms, 1 min) from epididymal vas deferens *in vitro* (Table 1).

Noradrenaline (10^{-7}–10^{-4} M) caused a dose-dependent contractile response in the epididymal vas deferens with an EC_{50} of 1.9 ± 0.5 μM (n = 5) and gave a maximal contraction of 1.58 ± 0.13 g tension (n = 5) at 10^{-4} M. Carbachol (10^{-7}–10^{-4} M) did not significantly modify the EC_{50} of noradrenaline or the maximal response of the epididymal vas deferens to noradrenaline (Table 2).

Table 1 Effect of carbachol and acetylcholine on the field stimulation evoked release of endogenous and labelled noradrenaline from epididymal vas deferens *in vitro*

	Fractional rate of release (% min^{-1})	
	[^{3}H]-noradrenaline	*Endogenous noradrenaline*
Control	0.52 ± 0.08 (4)	0.84 ± 0.06 (6)
Carbachol (4 μM)	0.47 ± 0.06 (4)	0.87 ± 0.08 (3)
Acetylcholine (10 μM)	0.43 ± 0.10 (3)	Not estimated

In determining the evoked release of [^{3}H]-noradrenaline, the vas deferens was stimulated at 70 V, 0.1 Hz, 1 ms, whereas the stimulation parameters of Oishi *et al.* (1983) were 70 V, 2.5 Hz, 0.5 ms for the study of endogenous noradrenaline release. The amount of noradrenaline released per minute is expressed as a percentage of total tissue content. Data are the mean ± s.e.mean of (n) experiments.

Table 2 Effect of carbachol on the contractions induced by noradrenaline in rat epididymal vas deferens

Drugs	*Noradrenaline induced contractions* EC_{50} (μM)	*Maximal contraction* (g tension)
Control	1.9 ± 0.5 (5)	1.58 ± 0.13 (5)
Carbachol 10^{-7} M	2.3 ± 0.6 (3)	1.50 ± 0.20 (3)
Carbachol 10^{-6} M	1.4 ± 0.3 (4)	1.46 ± 0.34 (4)
Carbachol 10^{-5} M	0.8 ± 0.4 (3)	1.68 ± 0.25 (3)
Carbachol 10^{-4} M	1.5 ± 0.3 (3)	1.45 ± 0.18 (3)

Results are the mean ± s.e.mean of (n) determinations.

Effect of prazosin on cholinergic potentiation

The direct contractile response to noradrenaline was antagonized by prazosin, an α_1-adrenoceptor antagonist (Figure 6a). At 10^{-8}–10^{-6} M, prazosin shifted the dose-response curves of noradrenaline to the right in a parallel and dose-dependent manner, and the pA_2 value was estimated to be 8.13 ± 0.09 (n = 3, Figure 7). At 1 μM, prazosin markedly attenuated the contractile response to field stimulation (2.5 Hz, 0.5 ms and 0.1 Hz, 1 ms) in the epididymal vas deferens (Figure 6a) but slightly potentiated that in the prostatic vas deferens (Figure 6b). The same concentration of prazosin, however, did not attenuate the potentiating effects of carbachol in either the epididymal (Figure 6a) or the prostatic (Figure 6b) vas deferens.

Discussion

The present study demonstrates that in addition to acetylcholine and carbachol (Graham *et al.*, 1968; Liao & Freer, 1983), other cholinomimetics including arecoline and pilocarpine potentiate the contractions to field stimulation in isolated preparations of rat vas deferens (Figure 3). Carbachol was particularly suitable for investigation because, unlike acetylcholine, it gave a stable potentiation and displayed minimal direct contractile effects on the vas deferens throughout the concentration-range tested (Figure 1). This action appeared to be mediated by muscarinic receptors since it can be competitively antagonized by atropine (pA_2 = 8.7, Figure 4) but not by hexamethonium (Liao & Freer, 1983).

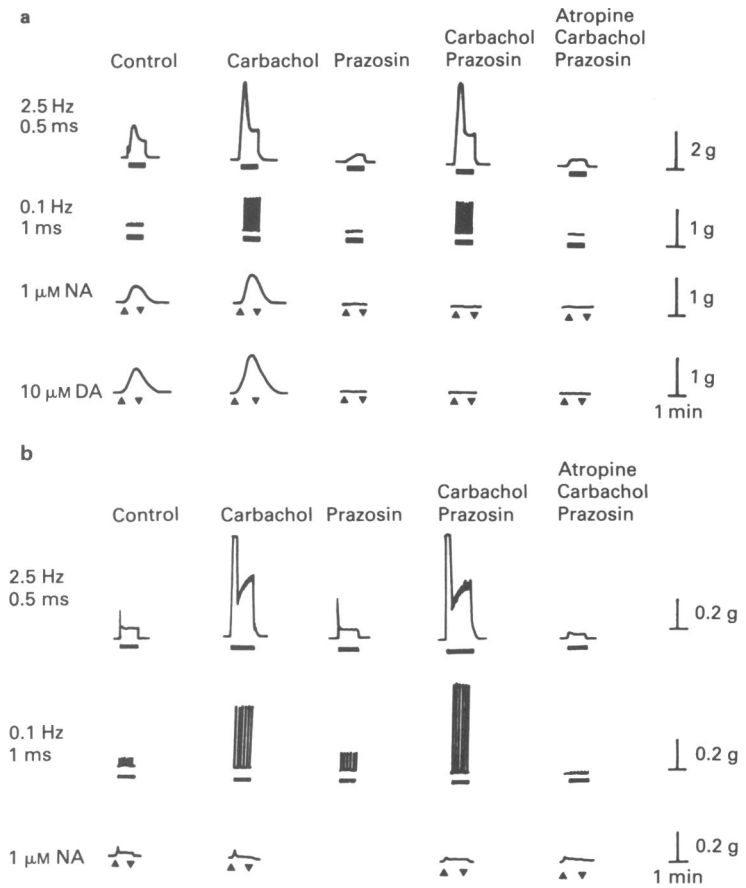

Figure 6 The effects of carbachol (4 μM), prazosin (1 μM) and atropine (5 μM) on the contractions to field stimulation (top panel: 2.5 Hz, 0.5 ms; second panel: 0.1 Hz, 1 ms) and the direct contractile effects of noradrenaline (NA) (1 μM) and dopamine (DA) (10 μM) in the epididymal (a) and prostatic (b) segments of rat vas deferens.

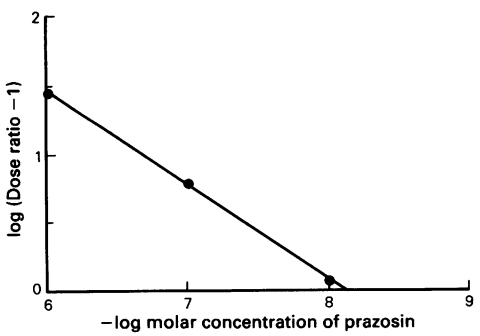

Figure 7 Arunlakshana & Schild (1959) plot to estimate the potency of prazosin as an antagonist of noradrenaline-induced contraction in rat vas deferens. Data shown are those of a typical experiment. $pA_2 = 8.1$.

There is pharmacological evidence suggesting that the motor innervation is not solely adrenergic in the rat vas deferens (Anton *et al.*, 1977; Brown *et al.*, 1983); however, at least in the epdidymal segment, transmission is predominantly adrenergic as its neurogenic contractions can be antagonized by α_1-adrenoceptor antagonists or by pretreatment of the animal with reserpine (McGrath, 1978). Our observation of a more pronounced potentiation by carbachol on the contractions to field stimulation in the epididymal vas deferens (Figure 2) had, therefore, led us to investigate the role of adrenergic transmission in this cholinergic action. Presynaptically, it was found that neither carbachol nor acetylcholine enhanced the field stimulation evoked release of noradrenaline from the epididymal vas deferens (Table 1). Postsynaptically, carbachol did not alter the contractions induced by exogenous noradrenaline (Table 2). Furthermore, the

presence of 1 μM prazosin which abolished the adrenergic contractile response in the rat vas deferens did not significantly alter the potentiating effect of carbachol on the contractile responses to field stimulation in either the epididymal or prostatic vas deferens (Figure 6a, b). These results suggest that the potentiating effect of cholinoceptor agonists on the contractions to field stimulation in the rat vas deferens was not via an enhancement of noradrenergic neurotransmission.

In the prostatic vas deferens, the neurogenic contractions were predominantly 'non-adrenergic' as they were resistant to adrenoceptor blockade (Figure 6b), and yet they could still be potentiated by carbachol (Figure 2). Furthermore, although the adrenergic component is dominant in the epididymal vas deferens, there is also some contribution from the 'non-adrenergic' component to the contractile response during field stimulation (Brown *et al.*, 1983). This is also evident in the present study as prazosin did not completely abolish the neurogenic contractions in the epididymal vas deferens (Figure 6a). Thus it is possible that cholinoceptor agonists might exert their potentiating effects on the contractions to field stimulation in the vas deferens through an enhancement of the 'non-adrenergic' transmission. Unfortunately in spite of the exquisite sensitivity to blockade by nifedipine (Blakely *et al.*, 1981; French & Scott, 1981, Brown *et al.*, 1983), the transmitter responsible for this 'non-adrenergic' component has not been unequivocally identified. More recently, however, it has been suggested that ATP acts as a co-transmitter with noradrenaline in the motor innervation of rat vas deferens (French & Scott, 1983; Sneddon *et al.*, 1984). This should provide a new lead for future evaluation of the mechanism underlying this potentiation by cholinomimetics.

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