Modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves

^{1,2}M.J. Rand & ¹C.G. Li

Department of Pharmacology, University of Melbourne, Victoria 3052, Australia

1 Acetylcholine-induced contractions of the rat isolated anococcygeus muscle were blocked by atropine (0.1 μ M), slightly enhanced by hexamethonium (0.1 mM) and tetrodotoxin (1 μ M), but little affected by prazosin (0.1 μ M).

2 In the presence of the α_2 -adrenoceptor agonist, UK14304, which raised the tone of the muscle, acetylcholine had a biphasic effect consisting of an initial relaxation followed by a contraction.

3 Atropine (0.1 μ M) enhanced the relaxant component and abolished the contractile component of the response, whereas tetrodotoxin, ω -conotoxin GVIA or hexamethonium abolished or greatly reduced the relaxant component.

4 The nitric oxide synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μ M) increased acetylcholine-induced contractions in the absence of UK14304 and markedly reduced the relaxant component to acetylcholine in the presence of UK14304. The effects of L-NAME were annulled by L-arginine (300 μ M).

5 The results suggest that acetylcholine acts concurrently on muscarinic receptors of the smooth muscle to cause contraction and nicotinic receptors of nitrergic nerves to cause relaxation. The observed response is the resultant of these two opposing effects and depends also on the prevailing tone.

Keywords: Acetylcholine; anococcygeus muscle; ω -conotoxin; hexamethonium; muscarinic actions; nicotinic actions; NANC transmission; N^G-nitro-L-arginine methyl ester (L-NAME); nitric oxide; tetrodotoxin

Introduction

The evidence that transmission at some neuroeffector junctions is mediated by nitric oxide (NO) has recently been reviewed (Rand, 1992a,b; Sanders & Ward, 1992; Sneddon & Graham, 1992). We have termed such transmission *nitrergic* (Rand & Li, 1990). Nitrergic transmission was first demonstrated for relaxant responses of rat and mouse anococcygeus muscle to stimulation of non-adrenergic non-cholinergic (NANC) nerves (Gillespie *et al.*, 1989; Li & Rand, 1989b; Ramagopal & Leighton, 1989; Gibson *et al.*, 1990).

The peripheral nitrergic nerves so far identified mainly belong to the parasympathetic system or, in the case of those in the enteric nervous system, have connections with the parasympathetic system. The anococcygeus muscles have a direct parasympathetic innervation (Gillespie & McGrath, 1973), and contain abundant acetylcholinesterase-positive fibres (Dail *et al.*, 1990), suggesting a cholinergic innervation. However, since acetylcholine does not mediate either motor or inhibitory transmission (Gillespie, 1972) the role of a cholinergic innervation is not clear.

Anococcygeus muscles respond to both muscarinic and nicotinic agonists. Muscarinic cholinergic receptors of the smooth muscle cells subserve contraction (Gillespie, 1972), and they are also present on both noradrenergic and nitrergic nerve terminals where they subserve inhibition of transmission (Rajani & Gulati, 1988; Li & Rand, 1989a). Nicotinic cholinergic receptors are present on both noradrenergic and nitrergic nerve terminals in the rat anococcygeus muscle, and nicotine acts on them to evoke release of the respective transmitters (Rand & Li, 1992).

The present study was carried out to determine whether responses to acetylcholine involve interactions with noradrenergic and/or nitrergic transmission in the rat anococcygeus muscle.

Methods

Male Sprague-Dawley rats (200–400 g) were killed by decapitation and the anococcygeus muscles were removed as described by Gillespie (1972) and set up in a 8 ml organ bath in physiological salt solution (PSS) of the following composition (mM): NaCl 118, KCl 4.7, NaHCO₃ 25, MgSO₄ 0.45, KH₂PO₄ 1.03, CaCl₂ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid, 0.14. The PSS was gassed with 5% CO₂ and O₂ and maintained at 37°C. The tone of the muscle was measured isometrically at a resting tension of 1 g wt. Field stimulation (1 ms pulses, 17 V cm⁻¹, at 1 to 5 Hz, for 10 s) was delivered from a Grass S88 stimulator through platinum wire electrodes on either side of the muscle. The isolated muscles were allowed to equilibrate for 30 to 45 min before making experimental observations. NANC-mediated relaxations were revealed by raising the tone and inhibiting contractile responses to noradrenergic nerve stimulation with the α_2 -adrenoceptor agonist, UK14304 (1 μ M) or guanethidine (10–30 μ M).

The effects of antagonists were studied after initial control responses to agonists had been obtained and the antagonists then remained in the PSS for the remainder of the experiment: corresponding agonist-induced responses without addition of an antagonist were elicited in the other anococcygeus muscle from the same donor rat to serve as a time control.

Differences between means were compared by Student's *t* test, and values of *P* < 0.05 were taken to indicate statistical significance.

Drugs

The drugs used were acetylcholine perchlorate (BDH, chemicals), L-arginine (Wellcome), atropine sulphate (Sigma), ω -conotoxin GVIA (Peninsula), guanethidine sulphate (Ciba), hexamethonium bromide (Sigma), N^G-nitro-L-arginine methyl ester (L-NAME, Wellcome), noradrenaline bitartrate (Sigma), prazosin hydrochloride (Pfizer), sodium nitroprusside (Sigma), tetrodotoxin (TTX, Sigma) and UK14304 (5-bromo-6-[2-imidazoline-2-Y1-amino]-quinoxaline; Pfizer).

¹ Present address: Pharmacology Research Laboratory, Department of Medical Laboratory Science, Royal Melbourne Institute of Technology, Victoria 3001, Australia.

² Author for correspondence (at 'Present address').

Results

Contractile responses to acetylcholine

Acetylcholine (1–1000 μM) elicited concentration-dependent contractions which were sustained during the period of exposure. The muscle relaxed promptly after washing out the acetylcholine. Subsequent responses to the same concentration were reproducible in magnitude and time course.

Atropine (0.1 μM) abolished responses to 1–300 μM acetylcholine (as shown for 100 μM acetylcholine in Figure 1) and greatly reduced (>90%) the response to 1000 μM acetylcholine.

The typical contractile response to a single concentration of acetylcholine (100 μM) is shown in Figure 1: it consists of an initial rapidly developing phase, which is complete between 20–25 s after exposure to acetylcholine, and a plateau which is reached after 3–4 min of exposure to acetylcholine. Hexamethonium (100 μM) increased the initial contractile response to acetylcholine, which rose more rapidly to a greater peak and then gradually fell to a plateau which was slightly greater than in the absence of hexamethonium (Figure 1 and Table 1).

Contractions elicited by acetylcholine (100 μM) were also slightly enhanced by tetrodotoxin (1 μM), but the effect of tetrodotoxin was most conspicuous in increasing the initial contractile response to acetylcholine (Figure 1 and Table 1).

In the resting drug-free state, field stimulation for 10 s periods at 1–5 Hz produced frequency-dependent, reproducible contractions. These contractions, as well as those induced by exogenously applied noradrenaline (1 μM), were abolished by the selective α_1 -adrenoceptor antagonist prazosin (0.1 μM) (results not shown). Prazosin (0.1 μM) had no significant effects on either the initial contraction or the plateau tension induced by acetylcholine (Figure 1 and Table 1).

Relaxant responses to acetylcholine

The adrenergic neurone blocking drug, guanethidine, raised the tone and reversed the response to field stimulation from a contraction to a relaxation, as first reported by Gillespie

(1972). The α_2 -adrenoceptor agonist, UK14304 (1 μM), also raised the tone and, like guanethidine, reversed responses to field stimulation from contractions to relaxations.

In the presence of UK14304, responses to acetylcholine (30–300 μM) contained contractile and relaxant components which were manifested in different proportions according to the concentration: with the lowest effective concentration (30 μM) the contraction predominated; then, with increasing concentrations, there was usually an initial transient relaxation followed by a contraction, both phases of the response being concentration-dependent (Figure 2a). Atropine (0.1 μM) abolished the secondary contraction and greatly enhanced the relaxant component of the response (Figure 2a).

In the presence of guanethidine, acetylcholine (100–1000 μM) produced only contractions and no relaxant component was evident. However, when the contractions were reduced by atropine (0.1 μM), a biphasic response was revealed, consisting of an initial relaxation followed by a contraction (results not shown).

Effects of L-NAME on responses to acetylcholine

L-NAME (100 μM) significantly enhanced contractions elicited by field stimulation, and also enhanced contractions elicited by acetylcholine as shown in Figure 1. The effect of L-NAME (100 μM) was most evident in the initial contractile response to acetylcholine, which rose more rapidly to a greater peak and then fell to a plateau which was slightly greater than in the absence of L-NAME (Figure 1 and Table 1). The response to acetylcholine was restored substantially to its initial form by 300 μM L-arginine (Figure 1 and Table 1).

In the presence of UK14304, L-NAME (100 μM) generally produced a small increase in tone and relaxations elicited by field stimulation (1–5 Hz for 10 s) were significantly reduced, as shown previously (Rand & Li, 1992). Relaxations elicited by acetylcholine in the presence of UK14304 were greatly reduced by L-NAME (Figure 2b), and the inhibitory effect of L-NAME on these relaxations was annulled by L-arginine (300 μM) (Figure 2b).

Effects of hexamethonium, tetrodotoxin and ω -conotoxin GVIA on acetylcholine-induced relaxations

The relaxant action of acetylcholine in the presence of UK14304 and atropine was abolished by hexamethonium

Figure 1 Responses elicited by exogenously applied acetylcholine (100 μM) in rat isolated anococcygeus muscles. Horizontal lines indicate the periods of exposure to acetylcholine. The contact time for atropine (Atrop, 0.1 μM), hexamethonium (C6, 0.1 mM), tetrodotoxin (TTX, 1 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), L-arginine (L-Arg, 300 μM) and prazosin (Praz, 0.1 μM) before eliciting responses to acetylcholine was 10 min. Similar results of each type were obtained in at least four separate experiments.

Table 1 Effects of various drugs on contractions of the rat anococcygeus to acetylcholine (100 μM)

Treatment	n	Initial phase tension (g)	Plateau phase tension (g)
Control	10	2.6 ± 0.3	3.7 ± 0.4
L-NAME (100 μM)	10	4.1 ± 0.6**	4.6 ± 0.6**
L-NAME + L-Arg (300 μM)	10	2.8 ± 0.4	3.8 ± 0.5
Control	5	2.6 ± 0.6	3.7 ± 0.6
Hexamethonium (100 μM)	5	3.9 ± 0.4**	4.6 ± 0.7**
Control	5	2.5 ± 0.5	3.5 ± 0.6
Prazosin (0.1 μM)	5	2.4 ± 0.4	3.4 ± 0.6
Control	9	2.4 ± 0.3	3.6 ± 0.4
Tetrodotoxin (1 μM)	9	2.8 ± 0.4*	3.7 ± 0.4
In the presence of prazosin (0.1 μM)			
Control	5	2.4 ± 0.3	3.6 ± 0.4
Tetrodotoxin (1 μM)	5	3.1 ± 0.5*	3.8 ± 0.7

The initial tension was calculated as the tension achieved in the first 20–25 s after the addition of acetylcholine (100 μM) and the plateau tension was that present after 4 min exposure of acetylcholine (see Figure 1).

* $P < 0.05$; ** $P < 0.01$: compared with control (paired *t* test).

Figure 2 (a) Effect of atropine (Atrop, 0.1 μM , contact time 10 min) on responses elicited by acetylcholine (ACh, 30, 100 and 300 μM) in a rat anococcygeus muscle in which the tone was raised by UK14304 (1 μM). (b) Effects of hexamethonium (C6, 0.1 mM), tetrodotoxin (TTX, 1 μM), ω -conotoxin GVIA (CTX, 50 nM) and N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), in the absence and presence of L-arginine (L-Arg, 300 μM) on the relaxations of rat anococcygeus muscles elicited by acetylcholine (100 μM). The contact time for C6, TTX, CTX, L-NAME and L-Arg was 10–20 min. The tone of the muscles was raised by UK14304 (1 μM) and all the experiments were conducted in the presence of atropine (0.1 μM). Columns represent mean values (with s.e.mean) ($n = 4-7$). * $P < 0.05$ (t test) compared with the corresponding time control (Con).

(100 μM) and significantly reduced by tetrodotoxin (1 μM) and by ω -conotoxin (50 nM) (Figure 2b).

Discussion

The autonomic innervation of the rat anococcygeus muscle contains noradrenergic, cholinergic and NANC components (Gillespie, 1972). Stimulation of sympathetic nerves causes noradrenergically mediated contractions (Gillespie, 1972), while stimulation of the NANC nerves produces a NO-mediated relaxation (for references, see Introduction).

Contractions of the rat anococcygeus muscle elicited by acetylcholine can be attributed to activation of postjunctional muscarinic receptors (Gillespie, 1972). They were blocked by atropine, but field stimulation-induced contractions were not affected by atropine and therefore do not contain a cholinergic component.

In addition, to its contractile action, the results obtained in the present study indicate that acetylcholine can also relax the anococcygeus if there is pre-existing tone. The relaxant action of acetylcholine is increased in the presence of atropine. On the other hand, the relaxation induced by acetylcholine was blocked by the nicotinic receptor antagonist hexamethonium, suggesting that it resulted from activation of nicotinic receptors. The transient nature of the relaxant action by acetylcholine could be due to its desensitization of nicotinic receptor (Rand & Li, 1992). The findings that the relaxations elicited by acetylcholine were also blocked by tetrodotoxin, the neuronal N-type calcium channel blocker ω -conotoxin GVIA, and by L-NAME, and

that the blocking effect of L-NAME was annulled by L-arginine, suggests that acetylcholine activates nicotinic cholinergic receptors on nitrergic nerves resulting in release of NO or a NO-yielding substance, as we have previously demonstrated with nicotine (Rand & Li, 1992).

The effect of atropine in enhancing the relaxant action of acetylcholine is presumably largely due to blockade of the counteracting contractile action exerted directly on the smooth muscle. An additional factor that may contribute to the enhancement is blockade of prejunctional muscarinic receptors associated with the nitrergic nerve terminals that subserve inhibition of transmission (Li & Rand, 1989a). Similar dual effects of acetylcholine in activating nicotine receptors to produce transmitter release and muscarinic receptors to inhibit it have been noted at noradrenergic nerve terminals (Löffelholz *et al.*, 1967; Lindmar *et al.*, 1968).

Activation of nicotinic cholinergic receptors on sympathetic nerve terminals in the rat anococcygeus also results in release of transmitter noradrenaline (Rand & Li, 1992); however, a nicotine-like effect of acetylcholine on sympathetic nerve terminals was not detected under the conditions used in the present study since acetylcholine-induced contractions were not affected by the α_1 -adrenoceptor antagonist, prazosin, which blocked responses to noradrenaline and stimulation of noradrenergic nerves. Alternatively, any change in the release of noradrenaline that acetylcholine may have produced was too small to contribute functionally to the contraction.

Contractions elicited by acetylcholine were enhanced by L-NAME, and this effect of L-NAME was annulled by L-arginine, which suggests that contractile responses to acetylcholine under resting conditions (without pre-existing tone) may actually contain a counteracting nitrergic component. In accord with this, acetylcholine-induced contractions were slightly enhanced by hexamethonium and tetrodotoxin, which indicates that the nicotine-like effect of acetylcholine is selective activation of nitrergic rather than noradrenergic transmission.

Recently, after completion of the present study, it was reported that L-NAME and other esters of arginine are muscarinic receptors antagonists and 100 μM of L-NAME was apparently effective in competitively inhibiting acetylcholine-induced contractions of endothelium-denuded rings of rabbit coronary artery and strips of circular smooth muscle from the dog colon (Buxton *et al.*, 1993). Although we have not investigated the possibility that such an action of L-NAME also occurred in the anococcygeus muscle, it is clear that even if it does occur it is far outweighed by the blockade of nitrergic mechanisms.

Activation of nitrergic transmission by acetylcholine may have a physiological significance in the control of the function of the muscle, since it has an abundant cholinergic innervation (Dail *et al.*, 1990). However, since the identification of a cholinergic innervation is based on the presence of nerve-associated cholinesterase, that enzyme might be present to prevent possible actions of acetylcholine.

Although a relaxant action of acetylcholine was clearly evident when UK14304 was used as a contracting agent, acetylcholine did not produce relaxations when guanethidine was used. We have shown previously that guanethidine inhibits nicotine-induced relaxation in the rat anococcygeus muscle (Rand & Li, 1992). Thus, the lack of a relaxant action of acetylcholine in the presence of guanethidine is due to attenuation of its nicotinic action; however, in the presence of both guanethidine and atropine, when the muscarinic contractile action of acetylcholine was blocked, the relaxant action of acetylcholine emerged.

The results obtained in the present study indicate that, in addition to its muscarinic smooth muscle-contracting action, acetylcholine also has a nicotinic relaxant action mediated by a nitrergic mechanism. There is a widespread distribution of nitrergic nerves (for references to reviews, see Introduction), and smooth muscle in most of the tissues innervated by them are responsive to muscarinic agonists. If the muscarinic

agonists are also nicotinic agonists, as is often the case, many of the actions of such agonists may need to be re-evaluated to determined whether the findings have been confounded by a contribution arising from activation of the nitrenergic nerves.

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(Received June 14, 1993
Revised August 19, 1993
Accepted September 1, 1993)