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Is co-transmission involved in the excitatory responses of the rat anococcygeus muscle?

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1 Electrical and mechanical responses to field (transmural) and extrinsic nerve stimulation were recorded simultaneously in the rat anococcygeus muscle. Membrane potential changes recorded intracellularly following either method of stimulation were indistinguishable. Single stimuli usually produced a slow depolarization; trains of pulses produced a fast excitatory junction potential (e.j.p.) initially, followed by a slow depolarization similar to that produced by single pulses. The fast e.j.ps, the slow depolarizations and the accompanying contractions were abolished by the α-adrenoceptor antagonists, phentolamine (1×10^{-6} M) or prazosin (1×10^{-7} M) and by tetrodotoxin (TTX, 1×10^{-6} M) but unaffected by α, β-methylene adenosine triphosphate (α, β-MeATP, $1-10 \times 10^{-6}$ M), an agent known to desensitize purinoceptors.

2 Application of noradrenaline (NA, $1 \times 10^{-8}-1 \times 10^{-6}$ M), by pressure ejection from a micropipette, depolarized the membrane and produced a localized contraction, both of which were abolished by phentolamine (1×10^{-6} M) or prazosin (1×10^{-7} M).

3 Application of adenosine-5′-triphosphate (ATP, $1 \times 10^{-4}-1 \times 10^{-3}$ M), by pressure ejection from a micropipette, produced a small membrane depolarization and localized contraction which were unaffected by phentolamine (1×10^{-6} M) or prazosin (1×10^{-7} M) but abolished by α, β-MeATP (1×10^{-6} M).

4 The results show that, in the rat annococcygeus muscle, (1) field or extrinsic nerve stimulation released only one excitatory transmitter, namely NA, although receptors for both NA and ATP were present on the muscle, (2) α, β-MeATP was selective for purinoceptors and (3) there was no evidence for excitatory co-transmission in this tissue.

Introduction

Electrical recording has provided evidence for two opposing hypotheses to account for the ineffectiveness (Holman & Surprenant, 1980) of α-adrenoceptor antagonists in blocking excitatory junction potentials (e.j.ps) produced by stimulation of peripheral sympathetic nerves. There is the view that while the e.j.p. is noradrenergically-mediated, it arises from an interaction between neuronally-released noradrenaline (NA) and a specialised postsynaptic junctional receptor, the γ receptor. On the other hand, exogenously added NA activates another, separate population of postsynaptic, α-receptors without causing detectable changes in membrane potential. This proposal has derived support exclusively from experiments on vascular smooth muscle (Hirst & Neild, 1981; Byrne & Large, 1986). Alternatively, resistance to α-adrenoceptor blockade may arise because two co-transmitters, adenosine-5′-triphosphate (ATP, or a related nucleotide) and NA are released following stimulation of sympathetic nerves, interaction of the nucleotide with its receptors accounting for the e.j.ps observed (Cunnane & Kirkpatrick, 1984; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984).

Much of the evidence for co-transmission has come from experiments using field (transmural) stimulation of isolated tissues (Meldrum & Burnstock, 1983; Sneddon & Westfall, 1984; Allcorn *et al.*, 1985a; Kennedy *et al.*, 1986). Field stimulation leads to the generation of propagated nerve impulses, as does extrinsic nerve stimulation, but it may also facilitate transmitter release by a local effect on those terminal varicosities already invaded by nerve impulses (Stjärne, 1977). Hyperpolarization or depolarization

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of nerve terminals following field stimulation, for example, may determine the extent to which facilitation and presynaptic inhibitory mechanisms influence transmitter release (Alberts *et al.*, 1981). Not surprisingly, the amount and hence the contribution of individual co-transmitters, released following field stimulation could be artefacts of the methods of stimulation employed, especially where recording has been made very close to the stimulating electrode.

Although evidence for co-transmission has come from many different types of experiments (e.g. Cheung, 1982; Sneddon & Burnstock, 1984; Allcorn *et al.*, 1985a) much of it, especially that involving sympathetic nerves, has relied heavily on the activities of two compounds, arylazidoaminopropionyl ATP (ANAPP$_3$) and α, β-methylene ATP (α, β-MeATP). Each of these compounds blocks P_2-purinoceptors selectively and so enables the role of the two transmitters in the neuronal response to be demonstrated. There remains the possibility, however, that these drugs could, in addition to their purinoceptor blocking effects, interfere with α-adrenoceptors, transmitter release or the ability of the postsynaptic membrane to generate e.j.ps. Indeed, a lack of specificity has been proposed and the suggestion made that, α, β-MeATP may abolish e.j.ps irrespective of whether they are produced by neuronally-released ATP or NA (Byrne & Large, 1986). Confirmation of this suggestion would seriously compromise the use of these drugs as investigative tools in co-transmission research.

Accordingly, the present study had two objectives; first to compare responses to field and extrinsic nerve stimulation and to determine, pharmacologically, whether e.j.ps elicited by either procedure differed. The second objective was to examine the selectivity of the ATP 'antagonist' α, β-MeATP for postsynaptic purinoceptors. The study was carried out *in vitro* on a tissue in which the e.j.p. is known to be mediated by NA acting on α-adrenoceptors, namely the rat anococcygeus muscle (Creed *et al.*, 1975).

Methods

Male Wistar rats (200–250 g) were stunned and bled. One anococcygeus muscle and attached extrinsic nerves (the genito-femoral and perineal branches of the pudendal nerves) were dissected out (McKirdy & Muir, 1978) and pinned onto the Sylgard base of a horizontal organ bath (2 ml approximately) at 36°C.

The caudal end of the muscle was attached by thread to a force displacement transducer (Grass FTO3C), and the other end passed through chlorided Ag/AgCl ring electrodes (o.d. 2 mm) for field stimulation and pinned to the base of the bath. The distance between the stimulatory and recording electrodes in field stimulated preparations was approximately 100–150 μm. Alternatively, each extrinsic nerve was passed through a ring electrode (o.d. 2 mm) so that the genito-femoral or perineal nerves could be stimulated either individually or simultaneously. Preparations were perfused (4–6 ml min^{-1}) with Krebs solution of the following composition (mM): NaCl 118.3, KCl 4.8, $MgCl_2$ 1.3, NaH_2PO_4 1.13, $CaCl_2$ 2.7, $NaHCO_3$ 25.0, glucose 11.2, pH 7.4, gassed with 95% O_2 and 5% CO_2.

Intracellular electrical measurements were made with capillary glass micro-electrodes (20–40 MΩ) filled with 3M KCl. Signals were passed to a unity gain high impedance amplifier (W.P. Instruments Model M4A), displayed on an oscilloscope (Tektronix D 11 dual beam storage) and u.v. recorder (EMI 8E6150 Mk II) and stored on an instrumentation tape recorder (Racal Store 4DS, Band width d.c. – 3.5 kHz).

NA and ATP were applied locally to the tissue from broken off micro-pipettes (approximate diameter 1–2 μm) by use of a pressure controlled micro-ejection device (Picospritzer, General Valve Corporation). Drugs were dissolved in a 0.9% NaCl solution, containing, in the case of NA, ascorbic acid (6×10^{-3} M), to prevent oxidation.

Four factors controlled the amount of drug reaching the recording electrode from the Picospritzer: (1) the diameter of the pipette tip: to ensure uniformity, the tip was broken back under microscopic control to 1–2 μm, (2) the distance of the pipette tip from the recording site: this was kept to within 1 mm, as measured with an eyepiece micrometer; (3) the ejection pressure: this was kept to between 40–50 psi; (4) the duration of the ejection (1–200 ms): this was varied as indicated in the text.

Statistics

Results are expressed as the mean ± s.e.mean of a number (n) of observations.

Drugs

The following were used: adenosine-5′-triphosphate (ATP, Sigma), α, β-methelene ATP (Sigma), idazoxan hydrochloride (Syntex), nifedipine (Sigma), (−)-noradrenaline bitartrate (Koch-Light), (±)-phentolamine mesylate (Ciba), (±)-prazosin hydrochloride (Pfizer), tetrodotoxin (Boehringer).

Drugs with the exception of nifedipine were dissolved in 0.9% NaCl solution, and this stock serially diluted with Krebs solution to the required concentration. Nifedipine was dissolved under sodium illumination in the minimum amount of Cremophor EL (Sigma) and diluted with Krebs solution. Cremophor EL itself was inactive. Drugs were perfused in the Krebs solution, except NA and ATP which were applied locally by pressure ejection. Concentrations in the text refer to the salt except tetrodotoxin, which was used as the base.

Results

Following the setting up of the preparation, the muscle was stretched to a tension of 0.75–1 g. Tension decayed over a 30 min equilibration period, to a lower value (approximately 0.5 g) which was maintained throughout the course of the experiments. Under these conditions, the muscle was electrically quiescent. The resting membrane potential ranged from −53 to −76 mV, with a mean of -63.7 ± 0.40 mV ($n = 170$).

Field ($n = 13$) and extrinsic ($n = 17$) electrical nerve stimulation (0.01–0.5 ms pulse width, 5–70 V) evoked a membrane depolarization and contraction, the amplitude of the depolarization depending on the stimulation parameters employed (Figure 1).

Following a single stimulus, by either method, a small slow membrane depolarization, measured as the time taken between 10 and 90% of the maximum voltage, with a latency of several hundred ms and a duration of several s was always recorded. The slow depolarization frequently appeared to follow the start of the mechanical event. This response will be termed the 'slow depolarization'. Short trains (2–5) of pulses at 5 or 10 Hz delivered by field or extrinsic nerve stimulation initially produced a 'fast e.j.p.' (rate of rise 15.0 ± 0.9 mV s^{-1}, $n = 49$) with a latency of less than 100 ms and a duration of under 1 s, followed by a slow depolarization, similar to that obtained to single pulses (Figure 1). The fast e.j.ps always preceded the onset of contraction. The rates of rise of the slow depolarization and the fast e.j.ps (Creed *et al.*, 1975) varied with frequency and stimulus strengths. This probably reflects facilitation of transmitter release, a feature characteristic of this muscle (Creed *et al.*, 1975). The mean ± s.e.mean rate of rise of the slow depolarizations measured under identical parameters of stimulation (1 pulse 0.2 ms, 20 V) was 0.6 ± 0.1 mV s^{-1} ($n = 12$).

In 3 out of 22 tissues, fast e.j.ps were absent and only slow depolarizations were evoked by either field or extrinsic nerve stimulation, even with longer trains of pulses (10) at supramaximal voltage (70 V). Conversely, in 2 out of 22 tissues, supramaximal field or extrinsic nerve stimulation, with single pulses, produced small (3–4 mV) fast e.j.ps, followed by a slow depolarization. Both the slow depolarizations and the fast e.j.ps were recorded in cells throughout the muscle, irrespective of their location, in response to field or extrinsic nerve stimulation. The electrical and mechanical responses to simultaneous stimulation of both the genito-femoral and perineal nerves were larger than those obtained to stimulation of either nerve separately (Figure 2).

The electrical responses produced by extrinsic nerve stimulation and those to field stimulation were identical. The fast e.j.ps, the slow depolarizations and the contractions produced by field (Figure 3) or extrinsic nerve stimulation (Figure 4) were abolished by phentolamine (1×10^{-6} M field stimulation $n = 6$, extrinsic nerve stimulation $n = 5$), prazosin (1×10^{-7} M field stimulation $n = 6$, extrinsic nerve stimulation $n = 8$) and by tetrodotoxin (TTX, 1×10^{-6} M, field stimula-

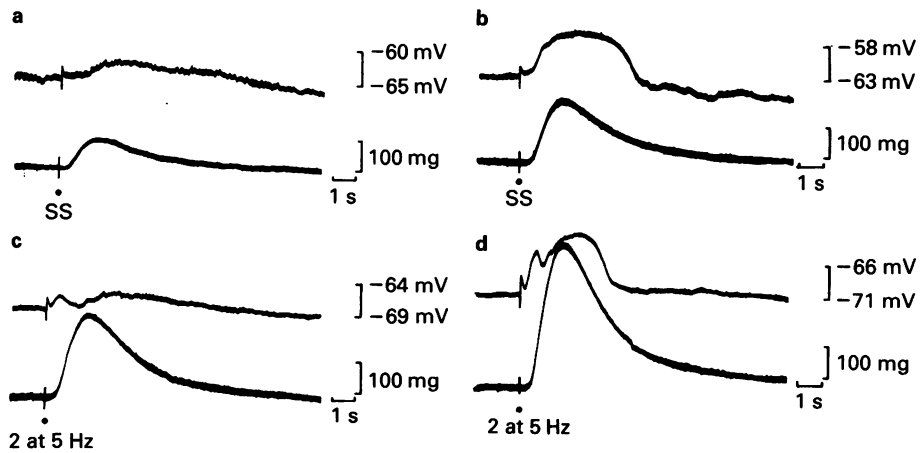

Figure 1 The effects of increasing stimulus strength on the simultaneously recorded electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to *field stimulation* of sympathetic nerves. Each panel shows, following the stimulus artefact, the responses to submaximal stimuli (a, b, single stimuli, SS 0.2 ms, 20 V left hand side, and 0.3 ms, 30 V; c, d, two stimuli 5 Hz, 0.1 ms, 7 V left hand side, and 0.1 ms, 12 V). The electrical response to a single stimulus was a slow depolarization. Following trains of pulses, a biphasic response was obtained. This consisted of an initial fast e.j.p. and a slow depolarization, the amplitude of each depending on stimulus strength. Intracellular electrical recordings were obtained from the same cell.

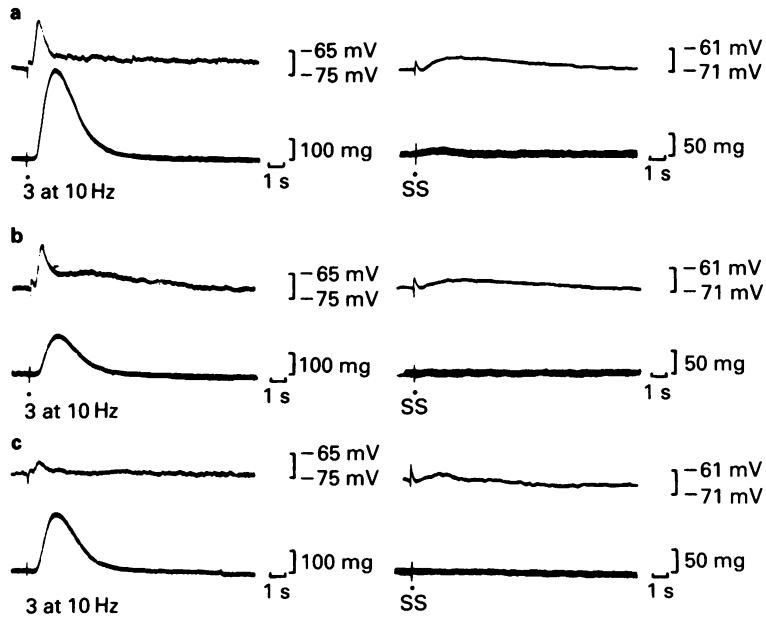

Figure 2 Simultaneously recorded electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to *extrinsic nerve stimulaton* (left hand column) 3 pulses, 10 Hz, 0.3 ms, 20 V; right hand column, single stimuli, 0.3 ms, 30 V. (a) Perineal and genito-femoral nerves together; (b) genito-femoral and (c) perineal nerves alone. Intracellular electrical recordings were obtained from the same cell. As with field stimulation (Figure 1), trains of pulses produced a biphasic response; an initial fast e.j.p. followed by a slower depolarization whereas single stimuli produced only a slow depolarization.

tion $n = 2$, extrinsic nerve stimulation $n = 2$). The stable analogue of ATP, α, β-MeATP ($1-10 \times 10^{-6}$ M) depolarized the preparation (field stimulated preparations $n = 7$; extrinsic nerve stimulated preparations $n = 6$) in a dose-dependent manner by up to 20 mV. This membrane depolarization had no significant inhibitory effect on the fast e.j.ps, the slow depolarizations or the contractions produced by either field (Figure 3) or extrinsic nerve (Figure 4) stimulation.

The absence of a depolarization resistant to α-adrenoceptor antagonists contrasted with events previously obtained in this tissue (Byrne & Large, 1984). To substantiate our findings, experimental conditions were used to optimise the possibility of demonstrating such e.j.ps if present.

In addition to the α_1-adrenoceptor antagonist, prazosin (1×10^{-7} M), idazoxan (1×10^{-7} M), which blocks presynaptic α_2-receptors, and thus the feedback inhibition on transmitter release, and nifedipine (1×10^{-6} M) at a dose which prevents muscle contraction without affecting synaptic potentials (Blakeley *et al.*, 1981) were used. Under these conditions, no postsynaptic depolarization was found even at high intensity stimulation parameters (5 pulses, 50 Hz, 0.01 ms, 70 V, Figure 5). These results therefore confirm the absence of non-adrenergic e.j.ps.

Pressure application of NA ($1 \times 10^{-8}-1 \times 10^{-6}$ M) produced a depolarization and a localized contraction which extended some 1–2 mm around the point of application. The characteristics of the membrane potential change varied with the amount of NA added (Figure 6). Low ($1 \times 10^{-8}-1 \times 10^{-7}$ M) concentrations of NA produced a slow depolarization, with a rate of rise of approximately 1 mV s^{-1} (1.5 ± 0.2 mV s^{-1}, $n = 8$), and a duration of several seconds. Higher ($1 \times 10^{-7}-1 \times 10^{-6}$ M) concentrations produced a more rapid depolarization (rate of rise 14.3 ± 1.1 mV s^{-1}, $n = 12$), with a duration of 1–2 s. Both fast and slow depolarizations were abolished by phentolamine (1×10^{-6} M Figure 7) or prazosin (1×10^{-6} M) but were unaffected by α, β-MeATP ($1-10 \times 10^{-6}$ M).

ATP ($1 \times 10^{-4}-1 \times 10^{-3}$ M) also produced small membrane depolarizations and localized contractions which were unaffected by phentolamine (1×10^{-6} M) or prazosin (1×10^{-7} M) but were abolished by α, β-MeATP (1×10^{-6} M). The rate of rise of the depolarization produced by ATP was graded with the concentration applied (3.8 ± 0.5 mV s^{-1}, $n = 12$); the depolarizations appeared uniform and there was no evidence of fast and slow components, as seen with NA (Figure 8).

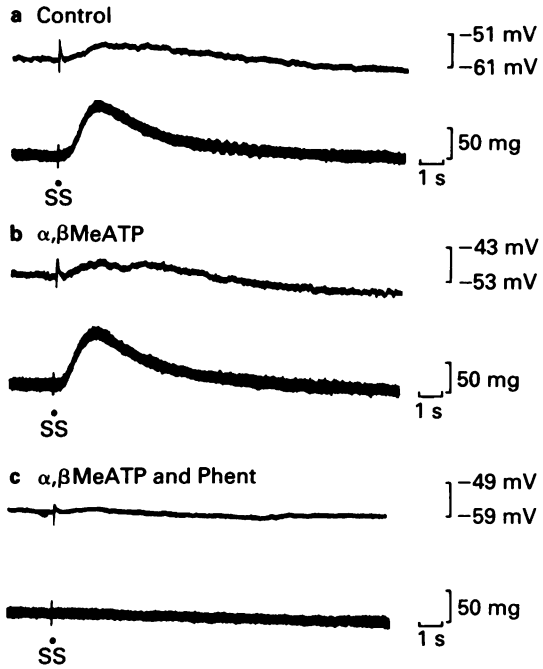

Figure 3 Effects of α, β-methylene adenosine triphosphate (α, β-MeATP) alone (1×10^{-6} M, b) and in the presence of phentolamine (Phent 1×10^{-6} M, c) on the responses of the rat anococcygeus muscle to submaximal *field stimulation* (single stimuli, 0.2 ms, 20 V); (a) control. Drugs were added to the perfusing fluid 20 min prior to recordings being made. In each panel the upper trace represents the electrical and the lower the mechanical recordings. Intracellular electrical recordings were made from 3 separate cells from the same preparation. Both the slow depolarization and the mechanical contraction were unaffected by α, β-MeATP but were abolished by phentolamine.

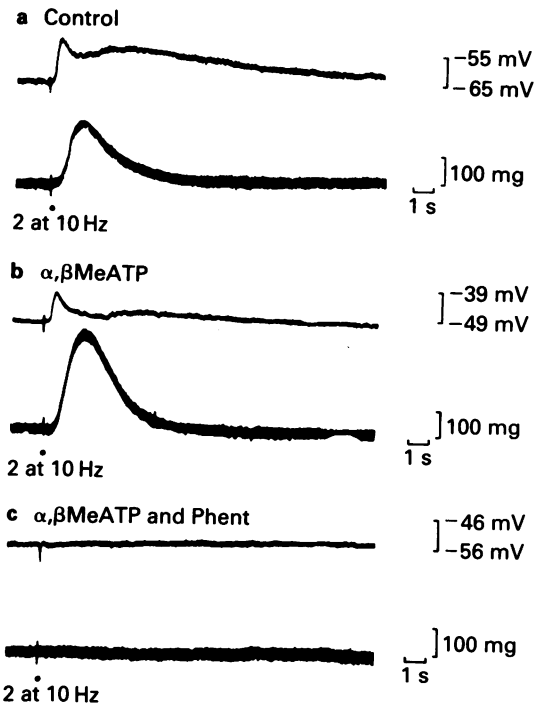

Figure 4 The simultaneous electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to stimulation of the genito-femoral and perineal nerves (2 stimuli, 10 Hz, (a) 0.3 ms, 20 V; (b) 0.3 ms, 15 V; (c) 0.3 ms, 50 V). Intracellular electrical recordings were made from 3 separate cells. The control response to extrinsic nerve stimulation (a) showed a fast e.j.p., a slow depolarization and a mechanical contraction. α, β-methylene adenosine triphosphate (α, β-MeATP, 1×10^{-6} M, b) enhanced the mechanical response and reduced, but did not abolish, the slow depolarization. The mechanical effects were due presumably to its ability to depolarize the membrane. The fast e.j.p., the slow depolarization and the contractions produced by *extrinsic nerve stimulation* were abolished by phentolamine (1×10^{-6} M, c). All drugs were perfused for 20 min before recordings were made.

Discussion

The possibility that the local effects of current produced by field stimulation could have released substances (e.g. ATP) directly from nerves rather than by action potential propagation in preterminal axons appears unlikely in the rat anococcygeus muscle preparation. ATP-mediated e.j.ps were not seen in response to either method of stimulation, although ATP-mediated responses sensitive to α, β-MeATP were observed following local application of the nucleotide. Moreover, there was no evidence that field stimulation facilitated transmitter release; e.j.ps following either method of stimulation were indistinguishable and were similar to those previously described in this tissue (Creed *et al.*, 1975).

Nor is there evidence from the present work that α, β-MeATP is non-selective in this tissue (cf. Byrne & Large, 1986). In the rat anococcygeus muscle, the e.j.p. was generated by the action of NA on α-adrenoceptors. That these receptors were of the α_1 variety is suggested by the effectiveness of the selective α_1-receptor antagonist, prazosin, as well as of phentolamine, which has affinity for both α_1-and α_2-adrenoceptors. In contrast, α, β-MeATP failed to inhibit either neuronally-released or locally-applied NA in the rat anococcygeus muscle. Together with the observation (Allcorn *et al.*, 1985b) that α, β-MeATP did not reduce transmitter release in the rabbit ear artery, the present results suggest that the drug is indeed selective for

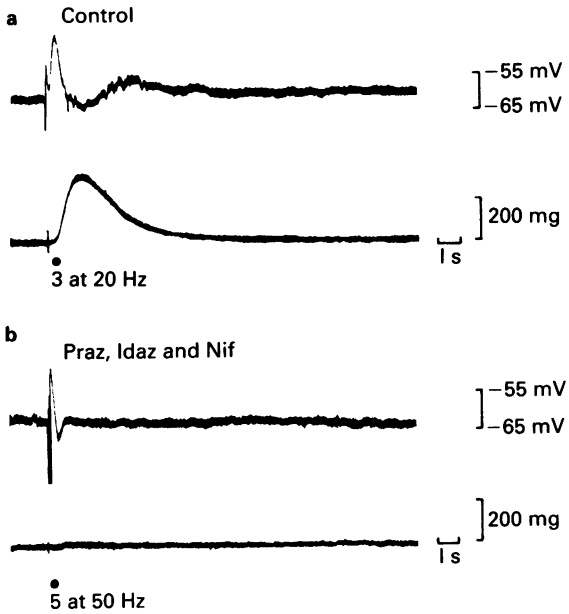

Figure 5 The electrical (upper trace in each panel) and mechanical responses of the rat anococcygeus muscle to *field stimulation* (a, 3 pulses at 20 Hz, 0.01 ms, 50 V; b, 5 pulses at 50 Hz, 0.01 ms, 70 V). Intracellular recordings were made from the same cell. In the control situation (a), field stimulation produced a fast e.j.p., slow depolarization and accompanying mechanical contraction. In the presence of prazosin (Praz, 1×10^{-7} M), idazoxan (Idaz, 1×10^{-7} M) and nifedipine (Nif, 1×10^{-6} M) (b) electrical and mechanical responses were abolished, and only a stimulus artefact remained even when both the number of pulses and the stimulus strength were increased. This confirms the exclusive noradrenergic nature of the neural response.

purinoceptors and does not modify postsynaptic effects of NA.

No support was obtained, from the present investigation, for the suggestion (Byrne & Large, 1984) that the fast e.j.p. was resistant to α-adrenoceptor antagonists. Pressure injection of NA produced not only slow (Byrne & Large, 1986), but also fast membrane depolarizations which, like the nerve-mediated responses, were sensitive to α-adrenoceptor blockade. ATP also depolarized the membrane, but only following ejection of high concentrations (1×10^{-4}–1×10^{-3} M).

The fast e.j.p. is not an enhanced or facilitated slow depolarization; both fast and slow membrane changes could be obtained in response to single stimuli. Both membrane responses were abolished by TTX, phentolamine or prazosin indicating they were mediated by neuronally-released NA acting on adrenoceptors. E.j.ps, in response to stimulation of either extrinsic nerve, were recorded from every cell impaled. Since there is the minimum of interaction between adjacent cells (Gillespie & Lüllmann-Rauch, 1974) and hence little electrical coupling, the ability to record e.j.ps in every cell probably results from the previously demonstrated (Gillespie & Lüllmann-Rauch, 1974) widespread distribution of sympathetic nerves in this tissue.

Unlike the fast e.j.p., which always preceded the onset of the contraction, the slow depolarization frequently appeared to follow the start of the mechanical event. This latter observation could indicate a voltage-independent contractile mechanism (for discussion see Bolton & Large, 1986) in this tissue. Alternatively the voltage threshold for contraction of the anococcygeus muscle may, as in the canine stomach fundus (Morgan *et al.*, 1981) lie close to the resting membrane potential. The initial membrane

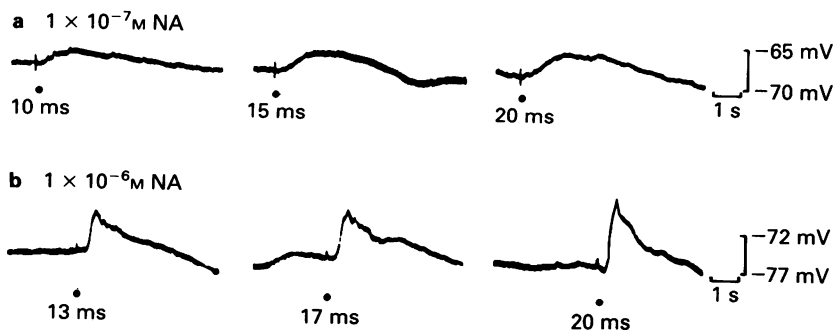

Figure 6 Intracellularly-recorded membrane potential responses of the rat anococcygeus muscle to micro application of noradrenaline (NA, 1×10^{-7} M, 1×10^{-6} M, in 2 separate cells, a and b) for increasing periods of time (10–20 ms). Micropipette tip diameter was 2 μm, and ejection pressure 40 psi. When applied locally close to the recording electrode, NA produced both fast and slow membrane depolarizations, depending on the concentration applied. Local mechanical contractions were also produced, the artefacts of which were often picked up by the recording electrode.

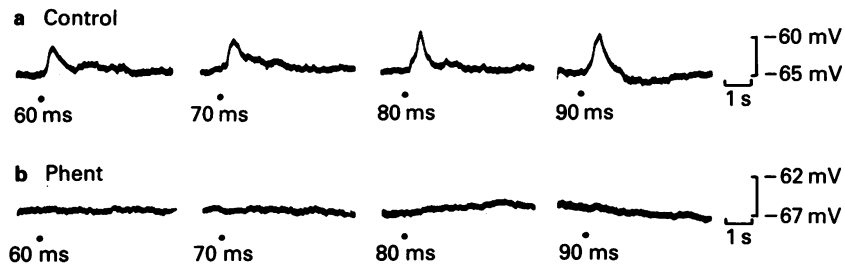

Figure 7 The effects of perfusing phentolamine (1×10^{-6} M for 20 min, b) on the membrane potential changes of the rat anococcygeus to local application of noradrenaline (NA, 1×10^{-7} M, ejection pressure 40 psi, micropipette tip diameter 2 μm) for the periods (ms) indicated. Local application of NA for short durations (60–90 ms) produced membrane depolarizations which were abolished by the α-adrenoceptor antagonist, phentolamine (Phent). Intracellular recordings were made from the same cell.

changes responsible for triggering contraction may, in consequence, have been too small to have been detected.

The two-component nature of the electrical membrane response to nerve stimulation resembled that already reported (Creed *et al.*, 1975; Byrne & Large, 1986), as did the physiological characteristics of the response itself. Except to establish their common transmitter origin, the function of each component of the depolarization was not investigated. There is, therefore, no evidence to deny the proposal (Byrne & Large, 1984) that the smaller, slow depolarization, the most common response to single stimuli, arises from the asynchronous release of transmitter while the faster, larger e.j.ps are the product of a synchronized release of transmitter following trains of stimuli. As previously shown in the field stimulated preparation (Creed *et al.*, 1975), both membrane depolarizations were susceptible to α-adrenoceptor blockade.

These results indicate that field or extrinsic nerve stimulation releases only one excitatory transmitter, although receptors for NA and ATP, both stimulant substances, are present (Gillespie, 1972). Each method of stimulation will release both excitatory and inhibitory transmitters however, since both types of fibres are present in each extrinsic nerve (McKirdy & Muir, 1978). The e.j.ps obtained are, therefore, the resultant of the effects of inhibitory and excitatory transmitters. These transmitters are contained in separate nerves (Gibson & Gillespie, 1973); chemical sympathectomy fails to abolish the inhibitory response. Nor is it likely that the slow inhibitory response seen during the e.j.ps in some cells, particularly at higher stimulation parameters (Figure 1c, d) represent slow i.j.ps. I.j.ps in the rat anococcygeus are mediated by non-adrenergic nerves (Creed *et al.*, 1975). The slow inhibitory response and the e.j.ps in the present work were abolished by the α-adrenoceptor antagonist,

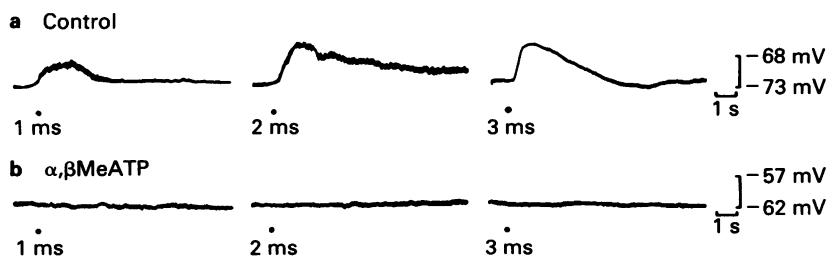

Figure 8 The effects of perfusing α, β-methylene adenosine triphosphate (α, β-MeATP, 1×10^{-6} M for 20 min, b) on the membrane potential changes of the rat anococcygeus muscle in response to local application of ATP (1×10^{-3} M, ejection pressure 40 psi, micropipette tip diameter 2 μm) for the periods (ms) indicated. Local application of ATP produced small membrane depolarizations which were abolished by α, β-MeATP. Intracellular electrical recordings were obtained from the same cell.

phentolamine. However, unlike the situation in other non-vascular (French & Scott, 1982; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984) and vascular (Cheung, 1982; Sneddon & Westfall, 1984; Allcorn *et al.*, 1985a; Kennedy *et al.*, 1986) smooth muscle, there was no evidence from the present experiments that the excitatory sympathetic nerves themselves exhibit functional co-transmission in the rat anococcygeus. It is interesting to note that there was no evidence for a purinergic component in the neuronally mediated excitatory response in this tissue, there were no e.j.ps resistant to α-blockade observed. This observation is in marked contrast to the e.j.ps in a wide variety of vascular and non vascular muscle which are clearly purinergic. We conclude therefore that the anococcygeus muscle preparation represents a tissue in which the sympathetic excitatory response is almost certainly noradrenergic.

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