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# Comparison of the biphasic excitatory junction potential with membrane responses to adenosine triphosphate and noradrenaline in the rat anococcygeus muscle

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**1** The effects of field stimulation and ionophoretic application of adenosine triphosphate (ATP) and noradrenaline were studied in the rat anococcygeus by means of an intracellular microelectrode.

**2** Field stimulation at room temperature produced three types of electrical membrane response: (a) a 'fast' excitatory junction potential (e.j.p.) which had a latency of less than 100 ms and a time to peak of 300 ms; (b) a 'slow' e.j.p. which had a latency of several hundred ms and a time to peak of 1–2 s, and (c) an inhibitory junction potential (i.j.p.) which had a time to peak of about 1.5 s. All three responses were blocked by tetrodotoxin.

**3** The ionophoretic application of ATP produced both monophasic and biphasic depolarizations; these responses had a latency of less than 30 ms and a time to peak of 150–300 ms. In contrast, ionophoretically-applied noradrenaline produced a depolarization which had a mean latency of 471 ms and a time to peak of 861 ms.

**4** The 'slow' e.j.p. and the noradrenaline-induced depolarization were blocked by prazosin whereas the 'fast' e.j.p. and the ATP responses were resistant to this antagonist and also to atropine.

**5** These results are further evidence that the 'fast' e.j.p. in some smooth muscle tissues is mediated by ATP.

## Introduction

In many smooth muscle preparations in which stimulation of sympathetic nerves produces a motor response, excitatory junction potentials (e.j.ps) recorded with an intracellular microelectrode are resistant to blockade with α-adrenoceptor antagonists, for example blood vessels (e.g. Holman & Surprenant, 1980; Hirst & Neild, 1980) and the vas deferens (Kuriyama, 1963; Burnstock & Holman, 1964). Recently Sneddon *et al.* (1982) and Sneddon & Westfall (1984) have suggested that in the guinea-pig vas deferens the e.j.p. is produced by adenosine triphosphate (ATP) which is released as a co-transmitter with noradrenaline from sympathetic nerves. In contrast, in the rat anococcygeus muscle neurotransmission appears to be typically adrenergic as field stimulation of the tissue evokes an e.j.p. which is blocked readily by phentolamine (Creed *et al.*, 1975). However, in some preliminary experiments with the rat anococcygeus it was noticed that the noradrenergic e.j.p. is preceded sometimes by a small depolarization (which will be termed the 'fast' depolarization) and the present experiments were undertaken to investigate the characteristics of this response. In particular an attempt was made to mimic the 'fast' e.j.p. with depolarizations produced by the ionophoretic application of ATP as it has been demonstrated that the time course of the noradrenergic e.j.p. and the response produced by ionophoretic application of noradrenaline is similar (Large, 1982).

## Methods

The isolated anococcygeus of the rat was set up for intracellular recording of membrane potential as described previously (Large, 1982; 1984). Muscles were superfused continuously with a modified Krebs solution which contained (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 11 and was bubbled with 5% $CO_2$–95%

$O_2$. Experiments were carried out at room temperature (20–23°C) because at temperatures higher than about 30°C spontaneous activity occurred which prevented stable intracellular impalements.

Membrane potentials were recorded with intracellular glass micro-electrodes filled with 4 M potassium acetate with resistances of 80–130 MΩ. Noradrenaline and ATP were applied by ionophoresis from similar micropipettes filled with the appropriate drug solution (concentration of 0.5 M) using a high voltage current pump (Purves, 1979). Noradrenaline and ATP were ejected with positive- and negative-going currents respectively. Nerve-evoked e.j.ps were produced by field stimulation (pulse width = 0.5 ms) of the tissue using either a partition chamber (Abe & Tomita, 1968) or two Ag-AgCl electrodes placed on either side of the preparation. Details of data recording and analysis have been described previously (Large, 1982). The bandwidth of the recording system was d.c. to 400 Hz.

The following drugs were used: noradrenaline bitartrate, adenosine 5′-triphosphate; phentolamine mesylate; prazosin hydrochloride; atropine sulphate; yohimbine hydrochloride; propranolol hydrochloride; α, β-methylene ATP and tetrodotoxin. The concentrations stated refer to the salt.

## Results

### *Excitatory junction potentials recorded in normal Krebs solution*

Field stimulation of the rat anococcygeus in normal Krebs solution at room temperature evoked three types of membrane response. The most common e.j.p. observed was characterized by a latency of several hundred ms between the stimulus artefact and the onset of depolarization (Figure 1a) and a total time to peak (latency + rise time) of about 1600 ms (Table 1). This response will be termed the 'slow' e.j.p. Sometimes a single stimulus produced no depolarization but larger e.j.ps could be obtained by stimulating the muscle for 150 ms at frequencies of 10–30 Hz (e.g. Figure 1b). Since it has been demonstrated previously in the anococcygeus muscle that e.j.ps evoked by short trains of pulses have a similar time course to e.j.ps evoked by a single pulse (Large, 1982) the former procedure was used to obtain responses of relatively large amplitude. Field stimulation of the muscle with more than one pulse produced such a powerful contraction that usually impalement was lost (e.g. Figure 1b and c). The membrane potential records might be distorted by mechanical move-

**Table 1** Characteristics of the time course of the membrane potential response produced by field stimulation or the ionophoretic application of noradrenaline or ATP

| | *Resting membrane potential* (mV) | *Response amplitude* (mV) | *Latency* (ms) | *Total time to peak* (ms) |
|---|---|---|---|---|
| 'Slow' excitatory junction potential | −59.5 ± 1.2 | 8.36 ± 1.08 ($n$ = 29) | 708 ± 21 | 1622 ± 34 |
| | | [a] [b] | | [a] [b] |
| Biphasic excitatory junction potential | −59.7 ± 1.3 | 3.50 ± 0.87 26.0 ± 3.17 ($n$ = 18) | —* | 313 ± 14 1558 ± 87 |
| [+]'Fast' excitatory junction potential (when not followed by a 'slow' e.j.p.) | −57.4 ± 1.9 | 5.44 ± 0.69 ($n$ = 37) | —* | 325 ± 16 |
| Ionophoretic noradrenaline | −65.6 ± 0.9 | 8.31 ± 1.51 ($n$ = 17) | 471 ± 21 | 861 ± 24 |
| Ionophoretic ATP | −63.7 ± 1.6 | 5.19 ± 0.48 ($n$ = 36) | —* | 267 ± 17 |

E.j.ps were evoked by a single stimulus or by applying two or three pulses as described in the text.
[+]Recorded from cells bathed in normal Krebs in the absence or presence of phentolamine $10^{-6}$ M.
[a] and [b] refer to the 'fast' and 'slow' depolarization respectively.
*Latency was sometimes obscured by stimulation artefacts but was always less than 100 ms.
The figures given are the mean ± s.e.mean of $n$ cells.

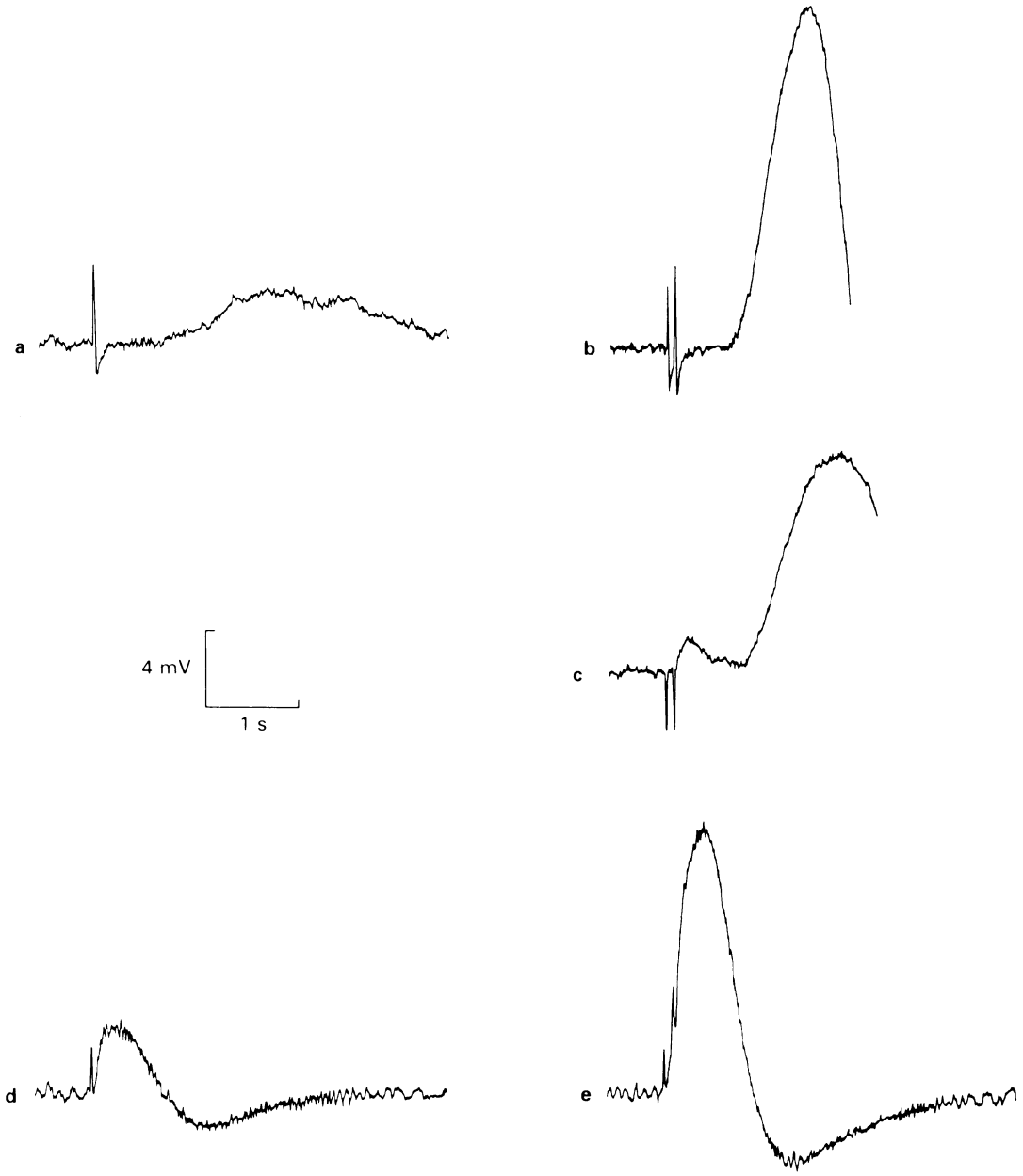


**Figure 1** Membrane responses produced by field stimulation in normal Krebs solution: (a) and (b) are 'slow' e.j.ps evoked by a single stimulus and 2 pulses at 10 Hz respectively. Resting membrane potential ($E_m$) = − 57 mV. (c) is a biphasic e.j.p. (2 pulses at 10 Hz) recorded from another muscle ($E_m$ = − 63 mV) and (d) (single shock) and (e) (2 pulses at 10 Hz) illustrate a 'fast' e.j.p. followed by an i.j.p. recorded in another preparation ($E_m$ = − 57 mV).

ments but it was found that the contractile response had not started before the peak depolarization had been reached. This result is in agreement with the findings of Creed *et al.* (1975) who showed that the time to peak depolarization of the e.j.p. was shorter than the latency of the contractile response. Thus it would seem likely that the measurements of latency and time to peak depolarization are not affected by mechanical artifacts. Moreover since the time to peak of the inhibitory junction potential (about 1500 ms) is similar to the time to peak of the slow e.j.p. (Table 1) the same argument applies.

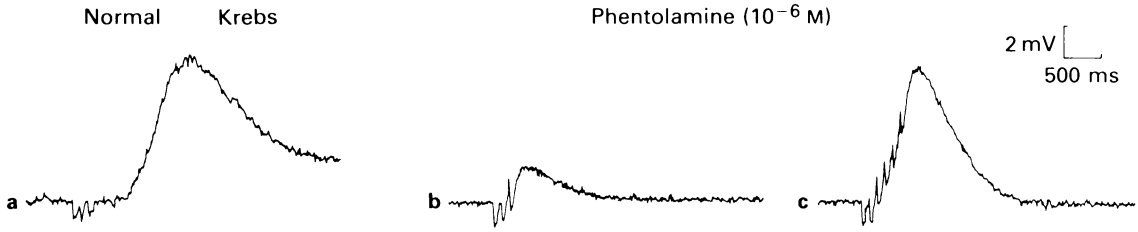


**Figure 2** Effect of phentolamine on the 'slow' e.j.p.: (a) (3 pulses at 10 Hz) was recorded from a cell in normal Krebs ($E_m = -63$ mV) and (b) and (c) were recorded in another cell ($E_m = -61$ mV) after the tissue had been bathed in phentolamine $10^{-6}$ M. (b) and (c) were evoked by applying 3 and 6 stimuli respectively at 10 Hz.

The second most common response to field stimulation was a biphasic e.j.p. (Figure 1c). The first ('fast') e.j.p. had no measurable latency and a mean time to peak of 313 ms. The second depolarization appeared to have a similar time course to the 'slow' e.j.p. as the mean time to peak was 1558 ms (Table 1) although its latency was not measurable because of the presence of the 'fast' e.j.p.

The amplitude of the 'slow' component of the biphasic e.j.p. was larger than that of the 'slow' e.j.p. recorded on its own (Table 1) for any given stimulation parameter. There is no obvious explanation for this observation although it may represent an increased transmitter release in the former situation.

On a few occasions (2 of 13 muscles) field stimulation produced a depolarization which had a time to peak of 325 ms (Table 1) but which was followed by a hyperpolarization rather than by the 'slow' e.j.p. (Figure 1d and e). Because of the similar time course it seems likely that the initial depolarization corresponds to the 'fast' e.j.p. seen in the biphasic excitatory responses. The secondary hyperpolarization had a time to peak of $1504 \pm 29$ ms (mean $\pm$ s.e.mean of 24 cells) and since it was recorded in the presence of

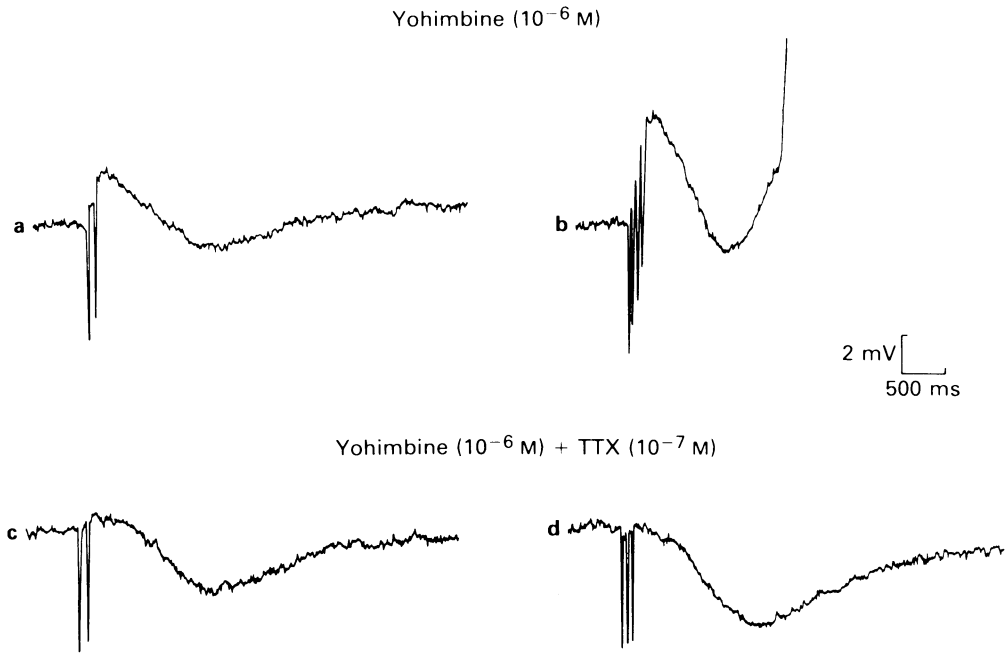


**Figure 3** Effect of tetrodotoxin (TTX) on the 'fast' e.j.p.: (a) and (b) were recorded in one cell ($E_m = -66$ mV) and (c) and (d) from another cell ($E_m = -57$ mV) after TTX ($10^{-7}$ M) had been added to the bathing solution. Yohimbine ($10^{-6}$ M) was present throughout. Stimulation parameters: (a) and (c), 2 pulses at 10 Hz; (b) and (d) 3 pulses at 20 Hz. Note that TTX $10^{-7}$ M abolishes the 'fast' e.j.p. with little effect on the i.j.p. which is blocked by higher concentrations of TTX.

phentolamine ($10^{-6}$ M) and atropine ($10^{-6}$ M) but was blocked by $10^{-6}$ M tetrodotoxin (TTX), this response probably represents the action of non-adrenergic non-cholinergic inhibitory transmitter which is well documented in this tissue (Creed *et al.*, 1975; Creed & Gillespie, 1977).

*Pharmacology of the 'fast' and 'slow' e.j.p.*

The 'slow' e.j.p. was blocked by low concentrations of $\alpha_1$-adrenoceptor antagonists and presumably corresponds to the e.j.p. described by Creed *et al.* (1975). However, an interesting observation was that in tissues where only the 'slow' e.j.p. was recorded in normal Krebs solution the addition of phentolamine $10^{-6}$ M not only blocked the 'slow' e.j.p. but caused the appearance of a 'fast' e.j.p. (compare Figure 2a and b). Presumably this effect of phentolamine (and yohimbine, see later) is due to a presynaptic action of increasing transmitter release in addition to a postsynaptic $\alpha$-receptor blocking effect. When the 'fast' e.j.p. was recorded in the presence of phentolamine there was little apparent muscle contraction and it was possible to obtain quite large 'fast' e.j.ps by increasing the number of pulses (Figure 2c). Yohimbine ($10^{-6}$ M), like phentolamine, increased the amplitude of the 'fast' e.j.p. although sometimes with this concentration of yohimbine a 'slow' e.j.p. and contraction were observed. Figure 3a and b show 'fast' e.j.ps recorded from one cell in $10^{-6}$ M yohimbine and Figure 3c and d were recorded from another cell in the presence of TTX $10^{-7}$ M (+ yohimbine) and the depolarization had been eliminated. Thus it is concluded that the 'fast' e.j.p. occurs as a consequence of nerve stimulation. Prazosin ($10^{-7}$ M) and atropine ($10^{-6}$ M) had no effect on the amplitude of the 'fast' e.j.p. Experiments were carried out on tissues which possessed no tone and thus the hyperpolarizations recorded in Figure 3c and d were not accompanied by mechanical relaxation. Moreover we did not study the inhibitory responses in tissues which possessed tone and so it is difficult to compare our data with those of Creed *et al.* (1975).

*Ionophoretic application of ATP and noradrenaline*

In view of the suggestion by Sneddon *et al.* (1982) and Sneddon & Westfall (1984) that the e.j.p. in the guinea-pig vas deferens is produced by the action of ATP it was of interest to see if the 'fast' e.j.p. in the rat anococcygeus could be mimicked by the ionophoretic application of ATP. The ionophoretic application of ATP produced a rapid depolarization which had a latency of less than 100 ms. Sometimes the responses produced by ATP were monophasic in shape (Figure 4a and b) but occasionally a biphasic depolarization was observed. This latter response usually occurred with large depolarizations, for example the depolarization in Figure 4c is about 16 mV whereas the amplitude of the responses in Figure 4a and b are about 2 and 6 mV respectively, although sometimes biphasic depolarizations were associated with smaller responses.

Details of the monophasic ATP-induced depolar-

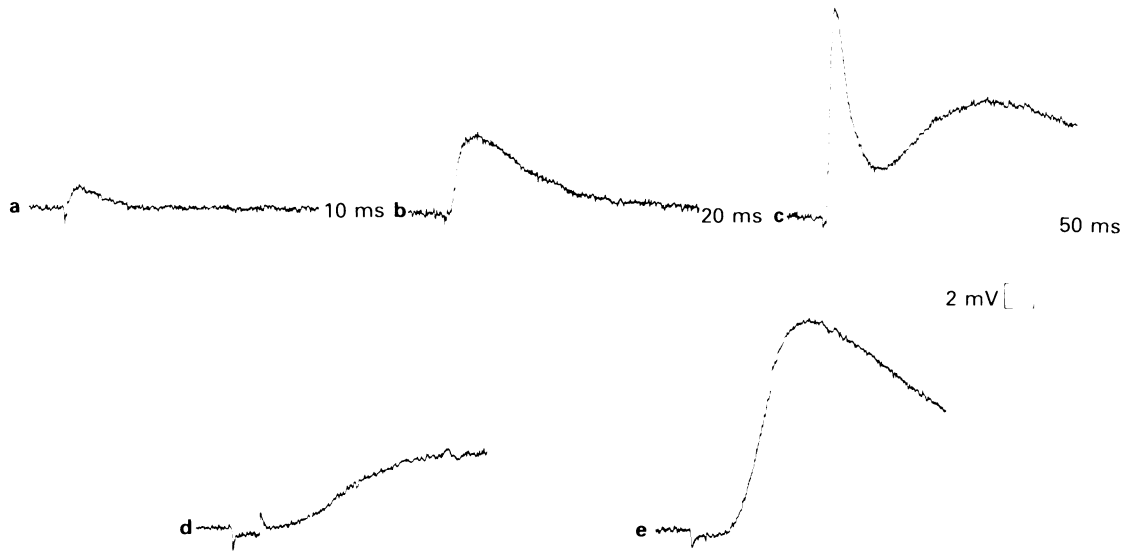


**Figure 4** Depolarizations produced by the ionophoretic application of ATP. All records from the same cell ($E_m = -66$ mV) and (d) and (e) are responses shown in (b) and (c) respectively but on a faster time base. Horizontal calibration: 200 ms for (a–c) and 20 ms for (d) and (e). Parameters of ionophoresis: 100 nA for durations shown at the right of the records.

izations are given in Table 1. With the biphasic responses the time to the first peak was 166 ± 20 ms (mean ± s.e.mean of 23 responses) and the time to the second peak was 839 ± 60 ms. Neither of these values correspond to the time of peak of the monophasic depolarizations but the impression obtained was that the initial response is an active response and the nature of these biphasic depolarizations will be discussed more fully later. A great technical difficulty was that the electrodes seemed to cease ejecting ATP, often after only one or two successful attempts even without movement of the electrode. Also with many electrodes no membrane response at all was observed. This was not a result of blocked electrodes as deduced from monitoring the voltage applied to the pipette tip. However the capricious nature of the ATP microelectrodes precluded any quantitative analysis with respect to tissue sensitivity to ATP before and after antagonists.

It was obvious that the latency of the ATP responses was small and Figure 4d and e are the responses shown in Figure 4b and c but on a rapid time base. The artefacts caused by the ionophoretic pulse can be seen quite clearly and the latency between the start of the ionophoretic pulse and the onset of depolarization is no more than 30 ms which presumably represents the upper limit of the latency of the ATP-induced depolarization. Therefore this response is much faster than that produced by noradrenaline which has a latency of several hundred ms (Large, 1982 and see Table 1 of this paper). The polarity of the voltage applied to the ATP microelectrode is such that direct electrotonic depolarization of the smooth muscle cells could occur but inspection of the records in Figure 4d and e show that this is not the case. Moreover, raising the ionophoretic electrode so that it was not touching the tissue produced responses of slightly smaller amplitude and noticeably slower time course; this observation is not consistent with direct stimulation of the cells but rather that the depolarizations are caused by the ionophoresis of ATP.

Depolarizations to ATP could be obtained using the normal parameters of ionophoresis (100 nA for 20–100 ms) from muscles bathed in solutions containing TTX, phentolamine and atropine (all at $10^{-6}$ M) so the responses were produced by the direct action of ATP on the smooth muscle, effects which

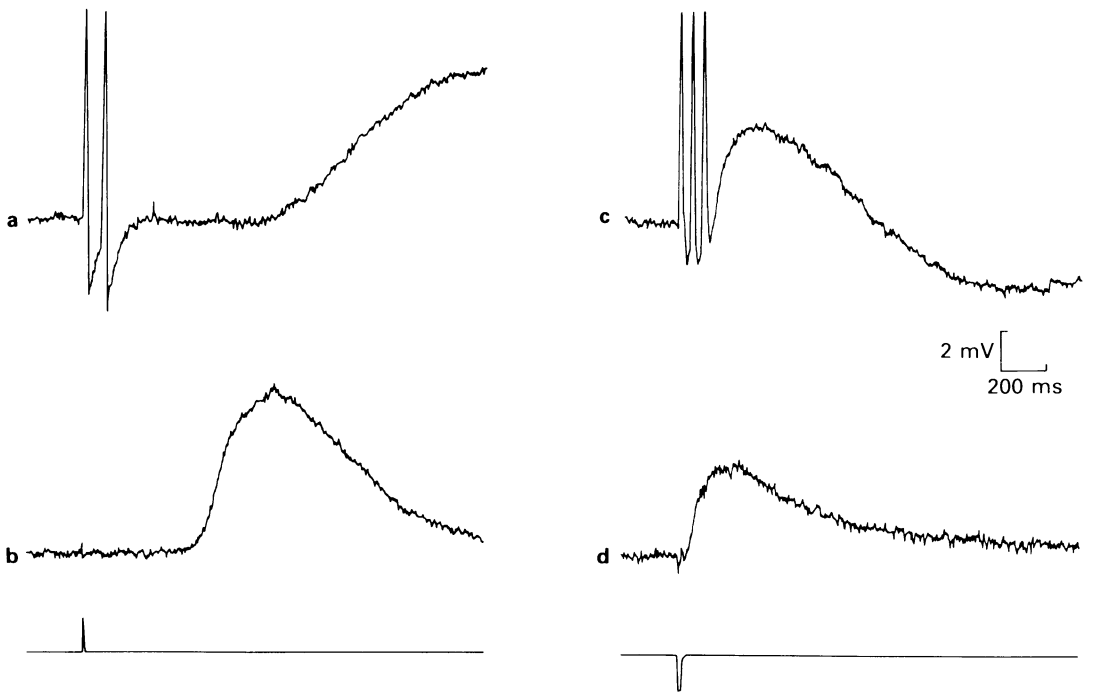


**Figure 5** Comparison of nerve-evoked responses with depolarization produced by the ionophoretic application of noradrenaline and ATP: (a) is a 'slow' e.j.p. evoked by 2 pulses at 10 Hz and the response (b) was produced by noradrenaline (2 ms: 100 nA) in the same cell ($E_m = -67$ mV); (c) is a 'fast' e.j.p. (3 pulses at 20 Hz) and (d) was elicited by ATP (20 ms: 100 nA) in another cell ($E_m = -57$ mV). (c) and (d) were recorded in the presence of phentolamine $10^{-6}$ M.

are not mediated by muscarinic receptors or adrenoceptors.

Experiments were carried out to match the 'slow' and 'fast' e.j.ps with the ionophoretic application of noradrenaline and ATP respectively. Figure 5a is a 'slow' e.j.p. evoked by two pulses (at 10 Hz) and Figure 5b is a depolarization produced by the ionophoretic application of noradrenaline. Essentially the results are similar to those found in the mouse anococcygeus muscle (Large, 1982) but are included for comparison with the ATP responses. The latency and the time to peak of the 'slow' e.j.p. is slower than that of the response to ionophoretically applied noradrenaline. This discrepancy was observed in the mouse anococcygeus (Large, 1982) but is even more marked in the rat. Figure 5c shows a 'fast' e.j.p. evoked by three pulses (at 20 Hz) and Figure 5d is a depolarization produced by the ionophoretic application of ATP onto the same cell. The large stimulus artefacts obscure the onset of the 'fast' e.j.p. but the overall time course of the two responses appear similar. A more complete analysis is shown in Table 1 where some characteristics of the time course of 'fast' and 'slow' e.j.ps and responses to ionophoretically-applied noradrenaline and ATP are compared.

There is no ATP antagonist which is readily available commercially but it has been reported that the stable analogue $\alpha,\beta$-methylene ATP antagonizes the responses to ATP in the vas deferens (Meldrum & Burnstock, 1983). However the application of $\alpha,\beta$-methylene ATP 2 $\mu$M produced a maintained depolarization and contraction in the rat anococcygeus and thus proved unsatisfactory in this tissue.

## Discussion

In the present study, field stimulation of the rat anococcygeus in normal Krebs solution evoked various types of membrane response: a 'fast' e.j.p. (sometimes followed by a hyperpolarization) a 'slow' e.j.p. or a biphasic e.j.p. ('fast' e.j.p. + 'slow' e.j.p.). Despite the fact that the time course of the 'slow' e.j.p. is significantly greater than the time course of the depolarization produced by ionophoretic application of noradrenaline, the pharmacology of this e.j.p. presented by Creed *et al.* (1975) and of the response to ionophoretically-applied noradrenaline (Large, 1982) suggest that the 'slow' e.j.p. is produced by the action of noradrenaline on $\alpha_1$-adrenoceptors. It is difficult to explain the discrepancy in the time course of the nerve-evoked and ionophoretically-induced depolarization and in fact the reverse situation might be expected i.e. the ionophoretic response being slower than the e.j.p. However, little is known about impulse conduction in the ground plexus and there may be temporal dispersion of transmitter release which could result in asynchronous action of transmitter on the smooth muscle cells.

In previous studies on the rat anococcygeus (Creed, 1975; Creed *et al.*, 1975) it was reported that field stimulation evoked two components of depolarization especially if the tissue was stimulated at high frequencies (10–100 Hz) but in those experiments both components of depolarization were blocked by phentolamine. In the present experiments the 'fast' e.j.p. was not antagonized by $\alpha$-adrenoceptor blocking agents and so probably does not correspond to either of the components observed by Creed *et al.* (1975). The apparent zero latency of the 'fast' e.j.p. might suggest direct stimulation of the muscle but because low concentrations ($10^{-7}$ M) of TTX abolished the response we conclude that the 'fast' e.j.p. results from nerve stimulation. The question arises whether the 'fast' e.j.p. in the anococcygeus muscle is caused by the action of ATP as has been suggested in the guinea-pig vas deferens (Sneddon *et al.*, 1982; Sneddon & Westfall, 1984). The 'fast' e.j.p. and the depolarization produced by ATP were similar in their resistance to phentolamine and atropine but the lack of an available ATP antagonist means that the pharmacological data are incomplete. There was extremely good agreement in the time course of the 'fast' e.j.p. and the monophasic ATP depolarizations with respect to negligible latency and rapid time to peak, highlighted by the very different time course of the noradrenaline-induced depolarization and the 'slow' e.j.p. At first sight the recording of biphasic ATP depolarizations would argue against ATP being the transmitter responsible for the 'fast' e.j.p. as biphasic 'fast' e.j.ps were never observed. However biphasic depolarizations in response to the ionophoretic application of noradrenaline have been recorded and yet there is little doubt that the 'slow' e.j.p. is produced by the action of noradrenaline. The biphasic nature of some of the depolarizations produced by the ionophoretic application of agonists may be related to the fact that the rat anococcygeus is capable of producing an 'active' membrane response (Creed *et al.*, 1975). By analogy with other electrically excitable tissues these active responses are more likely to occur when there is a rapid intense localized increase in membrane conductance which probably results when ATP is ionophoresed from a point source close to the muscle membrane but is less likely to occur in response to nerve stimulation. The presence of quinacrine-fluorescent nerve fibres in the rat anococcygeus and the increased release of ATP following field stimulation has been used as evidence that the inhibitory transmitter in this tissue may be ATP (Burnstock *et al.*, 1978). However, pretreatment of the muscle with indomethacin, a prerequisite for producing relaxation to ATP, is not required for

the observation of inhibitory responses to field stimulation. Moreover, in the present study, ATP depolarized cells which exhibited inhibitory junction potentials (mediated presumably by non-adrenergic non-cholinergic nerves) in response to field stimulation. The data from the present experiments give further support that the 'fast' e.j.p. in smooth muscle may be mediated by the action of ATP or a similar substance.

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