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ELECTROPHYSIOLOGICAL ANALYSIS OF THE INACTIVATION OF SYMPATHETIC TRANSMITTER IN THE GUINEA-PIG VAS DEFERENS

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SUMMARY

1. The properties of junction potentials evoked by nerve stimulation and by local application of drugs, and currents evoked by nerve stimulation, in the smooth muscle cells of the guinea-pig vas deferens have been investigated. The effects of temperature on these responses have been studied using intracellular and extracellular recording.

2. Local, brief (5–15 ms) application of 10^{-4} M-adenosine-5′-triphosphate (ATP) from glass micropipettes onto the surface of the vas deferens, using pressure pulses (103–206 kPa), elicited a depolarization of the smooth muscle cell membranes which closely resembled the nerve stimulation-evoked excitatory junction potential (EJP).

3. Local application of 10^{-4} M-noradrenaline (NA) failed to produce any detectable membrane potential response. Junction potentials elicited by a mixture of 10^{-4} M-ATP and 10^{-4} M-NA (ratio by volume 1:50) in the drug ejection micropipette were similar in shape to those evoked by ATP alone.

4. Cooling the tissue from 35 to 25 °C did not significantly alter resting membrane potentials but resulted in a significant prolongation of the rising and decaying phases of the EJPs. Fifty per cent decay times for EJPs at 35 and 25 °C were (mean ± S.D.) 236 ± 20 and 434 ± 30 ms respectively ($P < 0.01$).

5. Extracellularly recorded excitatory junction currents (EJCs) elicited by nerve stimulation, believed to reflect the transmembrane current underlying the EJPs, were prolonged in parallel at low temperatures (50% decay times of EJCs at 35 and 25 °C: 11·73 ± 3·94 and 26·15 ± 8·4 ms, respectively, $P < 0.01$).

6. Junction potentials evoked by locally applied, exogenous ATP were also significantly prolonged by cooling (50% decay times: 663 ± 88 ms at 35 °C and 1955 ± 79 ms at 25 °C, $P < 0.01$).

7. Bath application of 10^{-6} M-α,β-methylene ATP, the enzymatically stable, desensitizing analogue of ATP, reversibly abolished nerve-evoked EJPs. Local application of 10^{-6} M-α,β-methylene ATP led to a prolonged depolarization of the smooth muscle cells lasting between 20 and 60 s.

8. Junction potentials elicited by locally applied α,β-methylene ATP were not prolonged or otherwise significantly altered on cooling. The durations of the depolarizations were 46·0 ± 12·1 s at 35 °C and 43·4 ± 10·6 s at 25 °C ($P > 0.1$).

9. These results are consistent with the suggestion that ATP and not NA is the sympathetic neurotransmitter mediating the EJPs in the guinea-pig vas deferens. It is further proposed that an enzymatic inactivation process may be an important determinant of the lifetime of the purinergic transmitter mediating EJPs at this neuroeffector junction.

INTRODUCTION

The identity of the neurotransmitter(s) and the mechanisms of their action and inactivation in a number of sympathetically innervated tissues are undecided (Burnstock, 1986). The earlier assumption that noradrenaline (NA) is the only neurotransmitter mediating the electrical and contractile responses of tissues receiving a sympathetic innervation has been challenged by a number of observations. Recent evidence has led to the suggestion that ATP, co-released with NA from sympathetic neurones, may have a role as a neurotransmitter at these neuroeffector junctions, in some cases mediating those responses that are not explained by noradrenergic neurotransmission. In the rodent vas deferens, excitatory junction potentials (EJPs) and the twitch phase of contraction, which are resistant to the action of competitive α-adrenoreceptor antagonists such as prazosin and phentolamine (Ambache & Zar, 1971; McGrath, 1978), are blocked by the P_2-purinoceptor antagonist $ANAPP_3$ and the desensitizing agonist α,β-methylene ATP, and can be mimicked by the application of exogenous ATP (Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984; Allcorn, Cunnane & Kirkpatrick, 1986).

In order to accept ATP as a neurotransmitter, however, it is necessary to show that sympathetic nerves store and release ATP, and to establish its postjunctional action and mechanism of inactivation. Although there is evidence that ATP is present along with NA in sympathetic nerve vesicles (see Smith, 1972) and that endogenous ATP may be released from the nerves on electrical stimulation (Westfall, Stitzel & Rowe, 1978; Lew & White, 1987) and mediate postjunctional responses (Sneddon & Westfall, 1984), little is known about the mechanism of inactivation of this putative transmitter.

In the guinea-pig vas EJPs, which are believed to mediated by ATP released from sympathetic nerve terminals (Sneddon & Westfall, 1984), have been reported to be prolonged by cooling (Blakeley & Cunnane, 1982), suggesting the involvement of an enzymatic process in limiting the duration of action of transmitter in this tissue, as enzymes are known to be temperature sensitive. In this study we have investigated the membrane potential responses of these smooth muscle cells to the local application of ATP and its analogue, α,β-methylene ATP, which is resistant to enzymatic degradation, and the effect of temperature on these responses. Furthermore we have ascertained whether the prolongation of EJPs at low temperature is related to a prolongation of the underlying current events using a recently developed method of extracellular recording (Brock & Cunnane, 1987). A preliminary account of some of these findings has been communicated elsewhere (Cunnane & Manchanda, 1987).

METHODS

Male guinea-pigs (Hartley strain, 300–500 g) were killed by a blow to the head and bled and the vas deferens removed and pinned out in a 2 ml organ bath. A small area of the outer smooth muscle was exposed close to the stimulating electrode by removing the surface connective tissue. Intracellular and extracellular recordings, and local drug applications were made at the exposed site. The tissue was superfused at a rate of 2 ml/min with gassed (95% O_2, 5% CO_2) Krebs solution of the following composition (mM): NaCl, 118·4; KCl, 4·7; NaH_2PO_4, 0·4; $NaHCO_3$, 25·0; $CaCl_2$, 2·5; $MgCl_2$, 1·2; and glucose, 11·2.

The sympathetic motor nerves innervating the vas were excited by field stimulation of either the hypogastric nerve trunk or the prostatic end of the vas with rectangular voltage pulses, duration 0·1–0·5 ms, applied through insulated Ag–AgCl ring electrodes of separation 2 mm. Changes in membrane potential of smooth muscle cells on or near the surface of the tissue were recorded with intracellular glass microelectrodes filled with 5 M-potassium acetate, impedance 25–50 MΩ. The signals were amplified, low-pass filtered at 500 Hz (Neurolog NL 102 and 125, Digitimer), displayed on an oscilloscope and stored on an FM tape-recorder (Racal Store 4) for subsequent analysis. The temperature of the bath was monitored close to the site of recording with a thermocouple. In all experiments stimulation and drug-evoked responses at normal and low temperature were obtained from the same tissue, but not necessarily from the same cells because of the difficulty in maintaining intracellular penetrations, especially at low temperature. Several cells were, however, investigated in each tissue at the two temperatures, and results from several tissues subsequently pooled. Cooling was not observed to change the pH (7·3–7·4) of the bathing fluid.

Brief pulses of pressure (air) from a pressure application device (Picospritzer II, General Valve Corporation, U.S.A.) were used to apply locally small amounts of drug onto the surface of the vas deferens from the truncated tips (diameter 10–50 μm) of glass micropipettes. The amount of drug ejected from a given micropipette could be varied by changing either the duration or the intensity of the pressure pulse. The more accurate and reproducible method, that of varying the duration in millisecond steps (range 5–40 ms), was usually employed. The same micropipette was used throughout each experiment, thus eliminating pipette variability as a source of variation in evoked responses. The preparation was viewed under a binocular microscope and the recording electrode positioned within 150 μm–1 mm of the drug ejection pipette.

The criteria for 'successful' penetration of cells mentioned by Blakeley & Cunnane (1982) were strictly adhered to at all times, as it has been shown that very variable results are obtained if these are ignored, especially at low temperatures. Impalements were accepted only if they were abrupt, the membrane potential sealed to a more negative value, and spontaneous excitatory junction potentials (SEJPs) recorded during cooling.

Focal extracellular recording (see Brock & Cunnane, 1987, 1988) of spontaneous and evoked excitatory junction currents (EJCs) was performed using a bevelled glass electrode (tip diameter < 50 μm) filled with Krebs solution of the same composition as the bath solution and applied to the muscle surface with slight suction (seal resistance < 1 MΩ). The preparation was viewed on the stage of a Zeiss ACM microscope. Electrical activity was recorded through an AC amplifier (NL 104, low-frequency cut-off 0·1 Hz) and the output filtered (low pass, 1·5 kHz, NL 105) and recorded.

Drugs. Concentrated stock solutions of adenosine-5′-triphosphate (ATP; sodium salt), α,β-methylene ATP (sodium salt) and noradrenaline bitartrate (all chemicals from Sigma) were prepared and kept frozen until the time of use, when they were serially diluted to the required concentration in normal Krebs solution.

RESULTS

Effects of local application of ATP

The average resting membrane potential of the smooth muscle cells was 64 ± 5 mV (mean ± S.D.; range 56–80 mV, $n = 200$, fifteen preparations). In the absence of stimulation SEJPs of varying amplitude and time course were recorded, reflecting the quantal release of transmitter from sympathetic nerves (Burnstock & Holman, 1962; Cunnane & Stjärne, 1982). Stimulation of the postganglionic sympathetic

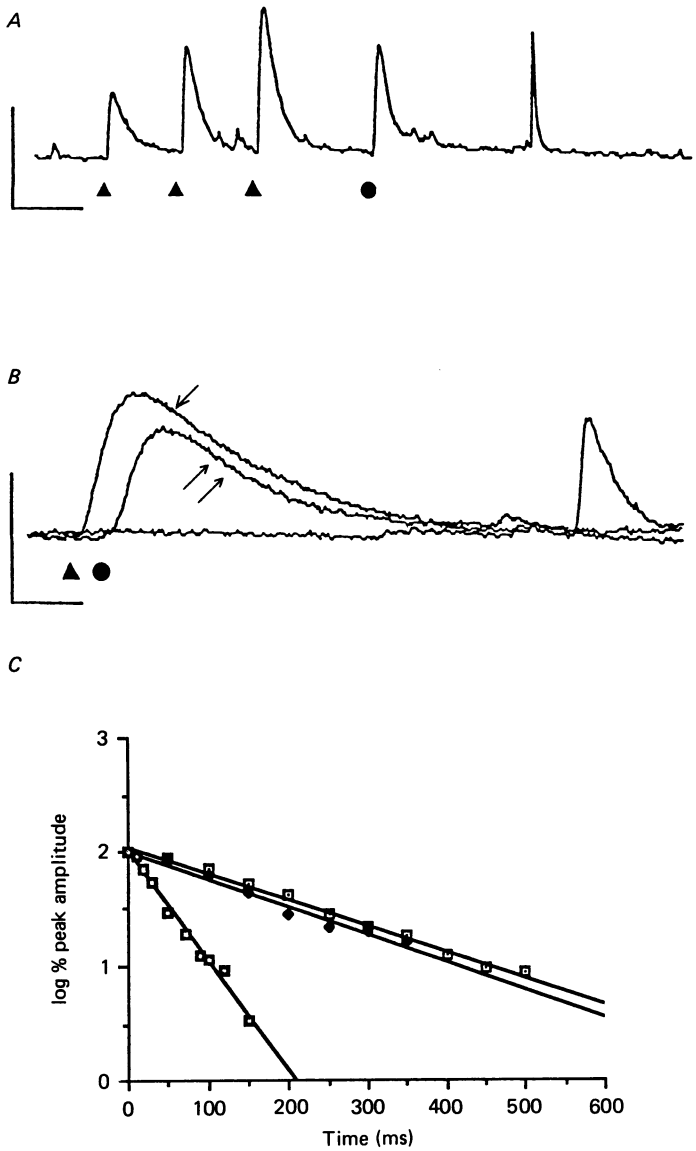

Fig. 1. Comparison of times courses of spontaneous and evoked junction potentials in the same cell of the guinea-pig vas deferens. EJPs were evoked by nerve stimulation (▲; 15 V, 0·5 ms) of the prostatic end of the vas with bipolar ring electrodes. ATP responses were obtained by local application of 10^{-4} M-ATP (●) onto the tissue surface by pressure ejection from a micropipette (pulse pressure, 103 kPa; pulse duration, 6 ms) placed ~ 100 μm from the recording microelectrode. *A*, Three EJPs showing facilitation followed by an ATP-evoked junction potential and an SEJP. *B*, superimposed traces on a faster time base to demonstrate the close similarity of the time course of the ATP response (double arrows) to the EJP (single arrow) but not the SEJP (far right). *C*, semilogarithmic plots of the decaying phases of the EJP (⊡), ATP response (◆) and SEJP (□). Calibration bars: *A*, 2 mV (vertical), 1 s (horizontal); *B*, 5 mV, 100 ms. Resting membrane potential: 65 mV.

nerves resulted in a more prolonged depolarization of the smooth muscle cells, the EJP lasting some 500–600 ms at 35 °C, which is due to the summed effect of transmitter released from numerous sites close to the recording electrode (Holman, 1970). Examples of both spontaneous and nerve-mediated evoked responses are shown in Fig. 1*A*.

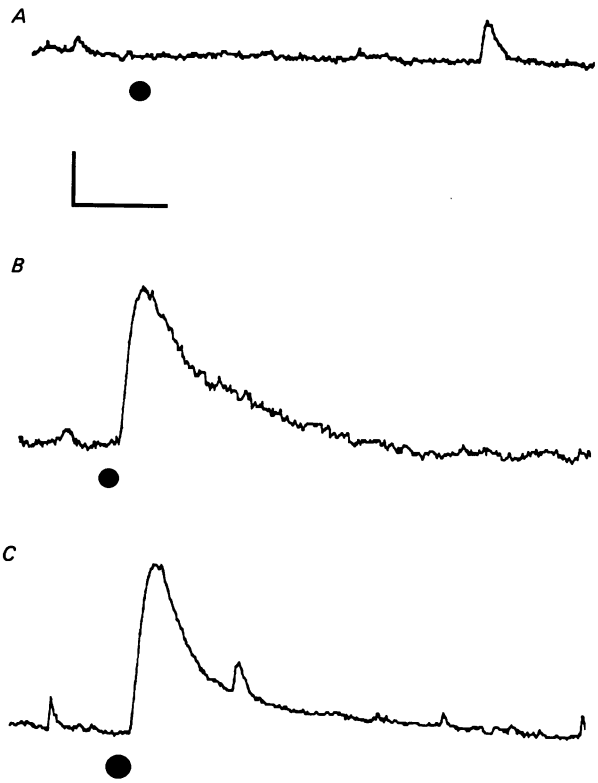

Fig. 2. *A*, lack of a membrane potential response of the smooth muscle cells of the guinea-pig vas deferens to the local application of 10^{-4} M-NA, in spite of the use of a long pulse duration (100 ms) and high pulse pressure (241 kPa). NA was applied from a glass microelectrode in a similar fashion to ATP (see Fig. 1) at the point indicated (●). *B* and *C*, junction potentials evoked in smooth muscle cells of the guinea-pig vas deferens by the local surface application of 10^{-4} M-ATP alone (*B*) or 10^{-4} M-ATP together with 10^{-4} M-NA (ratio by volume, 1 ATP : 20 NA) in the same micropipette (*C*) at the indicated points (○). The junction potentials had essentially similar rise times (80 ms), durations (1·5 s) and configurations. Resting membrane potential: 70 mV (*A*), 60 mV (*B*), 68 mV (*C*). Pulse pressure and duration: 138 kPa, 9 ms. Calibration bars: 2 mV, 200 ms.

Local, brief (pulse duration: 6 ms) application of 10^{-4} M-ATP onto the tissue surface resulted in a transient depolarization of the smooth muscle cells which mimicked the shape as well as time course of the stimulation-evoked EJPs (Fig. 1*A* and *B*). The correspondence between the time course of these two kinds of junction potentials was very close when care was taken to remove most of the connective tissue from the surface of the vas, and brief (5–10 ms) pulses of ATP were applied (Fig. 1*B* and *C*). In other experiments the ATP responses had a slightly longer

duration than that of EJPs, but the similarity in the shape of the potential changes was retained. It was not possible in the present experiments to mimic SEJPs in the same manner, conceivably because it is difficult to reproduce with exogenous drug application the localized release of transmitter (at the level of an individual nerve varicosity) which results in an SEJP (Cunnane & Stjärne, 1982; Brock & Cunnane, 1987).

Both EJPs and the depolarizations produced by exogenous ATP were capable of triggering muscle action potentials and subsequent contractions if the membrane potential change was large enough to reach threshold. With ATP responses the depolarizations needed for initiating contractions (15–20 mV) were usually lower than with EJPs (> 30 mV). This is most probably related to the fact that the ATP responses were recorded from cells some 0·3–1 mm distant from the site of application of ATP, and hence would be expected to be attenuated in magnitude at the recording site compared with the actual depolarization generated at the drug ejection site. Such an attenuation would be plausible if the membrane space constant of the guinea-pig vas is considered to be about 0·8 mm (Bywater & Taylor, 1980).

Local application of noradrenaline

Junction potentials could not be elicited with the local application of NA (10^{-4} M), despite the use of long pulse durations and high pulse pressure (Fig. 2*A*). However, the recording microelectrode was on occasion dislodged from the cell due to localized contractions evoked by exogenous NA in the absence of a preceding or accompanying depolarization. These contractions could be visualized under the microscope and occurred in the immediate vicinity of the drug ejection micropipette.

Junction potentials were however elicited when 10^{-4} M-ATP was present in the drug ejection micropipette along with 10^{-4} M-NA, even at a ratio by volume of 1 ATP: 50 NA (Fig. 2*C*). This ratio was used because estimates of the transmitter content of sympathetic nerve vesicles fall within the range 20–100 NA: 1 ATP (see Fredholm, Fried & Hedqvist, 1982). The depolarizations elicited were similar in shape and time course to the junction potentials evoked by local application of ATP alone (Fig. 2*B*), with no significant difference in rise times (ATP responses: 242 ± 51 ms; NA + ATP responses: 260 ± 48 ms, $n = 25$, $P = 0{\cdot}30$) or 50% decay times (ATP responses: 658 ± 92 ms; NA + ATP responses: 636 ± 111 ms, $n = 25$, $P = 0{\cdot}48$ (Student's paired t test)), indicating that NA does not measurably affect the electrical response of the smooth muscle cells to ATP.

Effects of cooling on EJPs

The effects of low temperature (21–25 °C) on the smooth muscle membrane properties of this tissue have been described in detail by Kuriyama (1964) and Blakeley & Cunnane (1982) and were confirmed by our observations. 'Successful' impalements (see Methods) during intracellular recording were much less frequently obtained on cooling, and the tissue was observed to contract more readily than at 35 °C. The frequency of occurrence and amplitudes of SEJPs were markedly reduced at lower temperatures, indicating some reduction in the postjunctional sensitivity to the transmitter.

The effect of temperature on the time course of EJPs is illustrated in Fig. 3. Figure

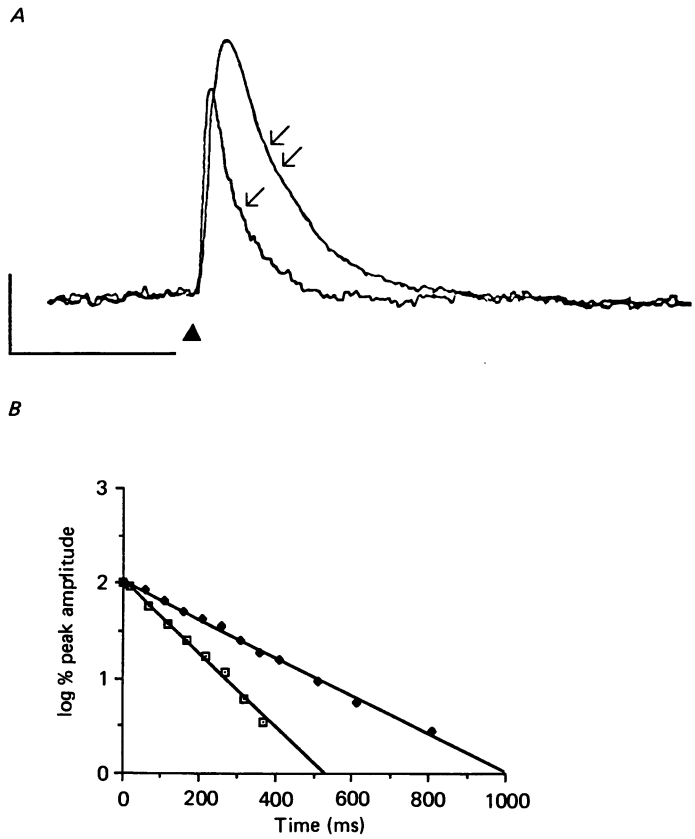

Fig. 3. Effect of temperature on EJPs in the guinea-pig vas deferens. *A*, prolongation of the nerve-evoked (▲) junction potential at 25 °C (double arrows) as compared with the control response at 35 °C (single arrow). The first EJPs in a train were taken for comparison. Facilitation of the first EJP in a train at lower temperature is similar to that described by Blakeley & Cunnane (1982). Stimulation parameters: 15 V, 0·3 ms. EJPs recorded from the same cell (resting membrane potential 62 mV). Calibration bars: 2 mV, 1 s. Rise time and 50% decay time for the illustrated records were as follows: 60 and 204 ms at 35 °C, 106 and 450 ms at 25 °C. *B*, semilogarithmic plot of the decay phase of the EJPs against time at 35 and 25 °C. ⊡: 35 °C; ◆: 25 °C ($r = 1{\cdot}00$ for both fits).

3*A* shows EJPs elicited at 35 and 25 °C, and Fig. 3*B* shows a comparison of the monoexponential decay phases of the depolarizations at the two temperatures in a semilogarithmic plot. Cooling the tissue from 35 to 25 °C did not significantly alter the mean resting membrane potential but EJPs were significantly prolonged (rise time and 50% decay time (ms): $52 \pm 4 \pm 4{\cdot}8$ and 236 ± 20 at 35 °C, $85{\cdot}4 \pm 10{\cdot}2$ and 434 ± 30 at 25 °C, $n = 36$, $P < 0{\cdot}01$ (Student's paired t test)).

Effects of cooling on EJCs

By applying a bevelled glass electrode to the surface of the vas, extracellular recordings can be made of the electrical response of the postjunctional smooth muscle membrane to spontaneous and evoked transmitter release (Brock & Cunnane, 1987,

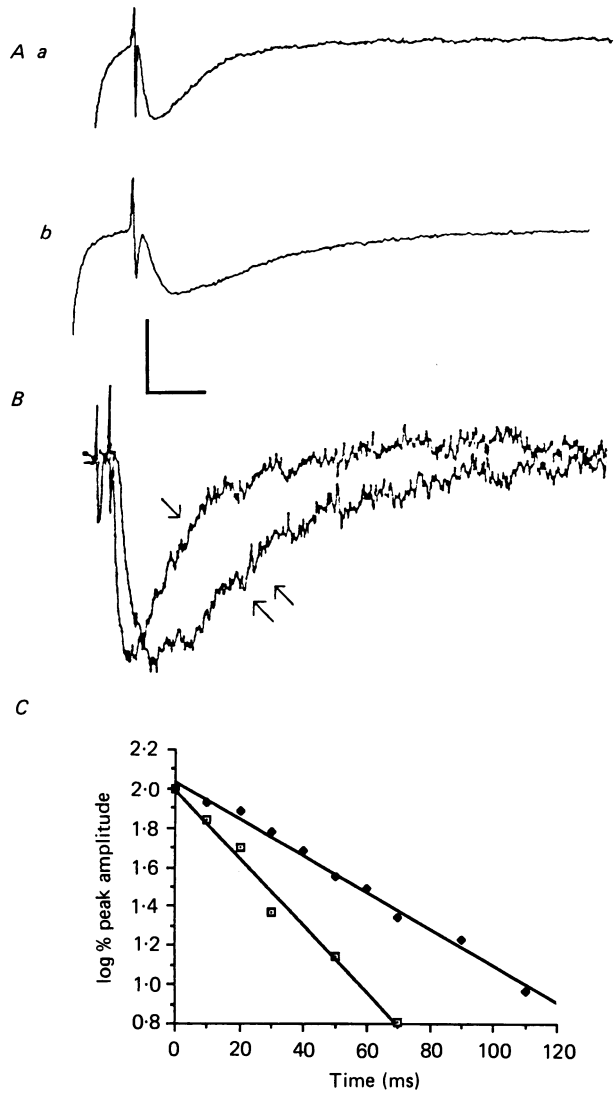

Fig. 4. Effect of temperature on EJCs recorded extracellularly in the guinea-pig vas deferens. The nerve terminal action potential preceding the EJCs is also shown. *A*, averaged EJCs (88 in *a*, 82 in *b*) at 35 °C (*a*) and 25 °C (*b*). EJCs in either case were selected from continuous trains of 200 stimuli at 1 Hz (stimulation parameters: 10 V, 0·16 ms). Only those records distinguishable from the background noise were chosen for averaging. This procedure eliminates any distortion in the averaged time course that might result from taking an indiscriminate average of all records, which would include many failures of release from the terminal regions of the sympathetic nerves, despite successful action potential invasion (see text). Note the change in latency from the point of stimulation, and concomitant broadening, of the nerve action potential at 25 °C. *B*, individual EJCs recorded at 35 °C (single arrow) and 25 °C (double arrow), taken from the same series of EJCs as used for the averaged records. Larger-than-average EJCs were taken for this illustration. Calibration bars: 20 μV, 10 ms. *C*, semilogarithmic plot comparing the monoexponential decay phases of the EJCs illustrated in *B*. ⊡: EJC at 35 °C (r for curve fit = 0·99); ◆: EJC at 25 °C (r for curve fit = 1·00).

1988). The negative-going SEJCs and EJCs thus recorded, exhibit the same pharmacological sensitivities as the intracellularly recorded SEJPs and EJPs; for instance they are unaffected by α-adrenoreceptor antagonists but are blocked by α,β-methylene ATP (Brock & Cunnane, 1988) which desensitizes and blocks postjunctional P_2-purinoceptors (Kasakov & Burnstock, 1983).

Extracellularly recorded EJCs, believed to represent the transmembrane current underlying the intracellularly recorded EJPs (Brock & Cunnane, 1987, 1988; Åstrand, Brock & Cunnane, 1988) had a 10–90% rise time and 50% decay time, of $3{\cdot}31 \pm 1{\cdot}27$ and $11{\cdot}73 \pm 3{\cdot}94$ ms (mean $\pm$ S.D., $n = 33$) respectively at 35 °C. On cooling, they were significantly prolonged (rise time and 50% decay time at 25 °C: $6{\cdot}95 \pm 3{\cdot}05$ and $26{\cdot}15 \pm 8{\cdot}84$ ms respectively, $n = 33$, $P < 0{\cdot}01$ (Student's paired t test)).

Figure 4*A* shows averages of eighty-eight and eighty-two traces at 35 and 25 °C, respectively, of EJCs evoked by sympathetic nerve stimulation recorded in the same attachment, with the nerve action potential preceding the junction currents in each case. EJCs were not observed in response to every stimulus, in spite of non-intermittent arrival of the nerve action potential in the sympathetic nerve terminals. For a more rigorous analysis, therefore, the averaging procedure was limited to those traces which exhibited EJCs distinct from the background noise. The average time course of the EJCs at 25 °C was clearly prolonged as compared with that at 35 °C. Figure 4*B* and *C* compare individual EJCs recorded at 35 and 25 °C. Both the latency from the point of stimulation as well as the 'area' of depolarization underneath the nerve terminal action potential are increased upon cooling (Fig. 4*A* and *B*). The possible effects on transmitter release of this phenomenon have yet to be elucidated. However, no significant difference was observed in the frequency of transmitter release at the two temperatures (as judged by the frequency of the EJCs), whilst the time course of the EJC was clearly affected.

The effect of temperature on ATP responses

If neuronally released ATP mediates EJPs and EJCs, then these effects of cooling might also be expected on responses to exogenously applied ATP. Figure 5*A* shows typical ATP responses at 35 and 25 °C, taken from separate cells. In parallel with the effects on EJPs, the membrane potential responses to exogenously applied ATP were similarly prolonged at lower temperatures (Fig. 5 and Table 1). The time courses of the exponential decay phase of ATP-evoked junction potentials at 35 and 25 °C are compared in a semilogarithmic plot in Fig. 5*B*. Table 1 includes data from eight tissues and demonstrates that it was possible to observed continuous prolongation of ATP responses with 5 °C increments of temperature.

Membrane potential response to α,β-methylene ATP

Bath application of the stable, desensitizing analogue of ATP, α,β-methylene ATP (Kasakov & Burnstock, 1983) has been reported to lead to a prolonged contractile response and subsequent abolition of ATP responses, both electrical and contractile, in the guinea-pig vas deferens (Sneddon & Westfall, 1984). Figure 6 shows the time course of the desensitizing effect of 10^{-7} M-α,β-methylene ATP on nerve-evoked

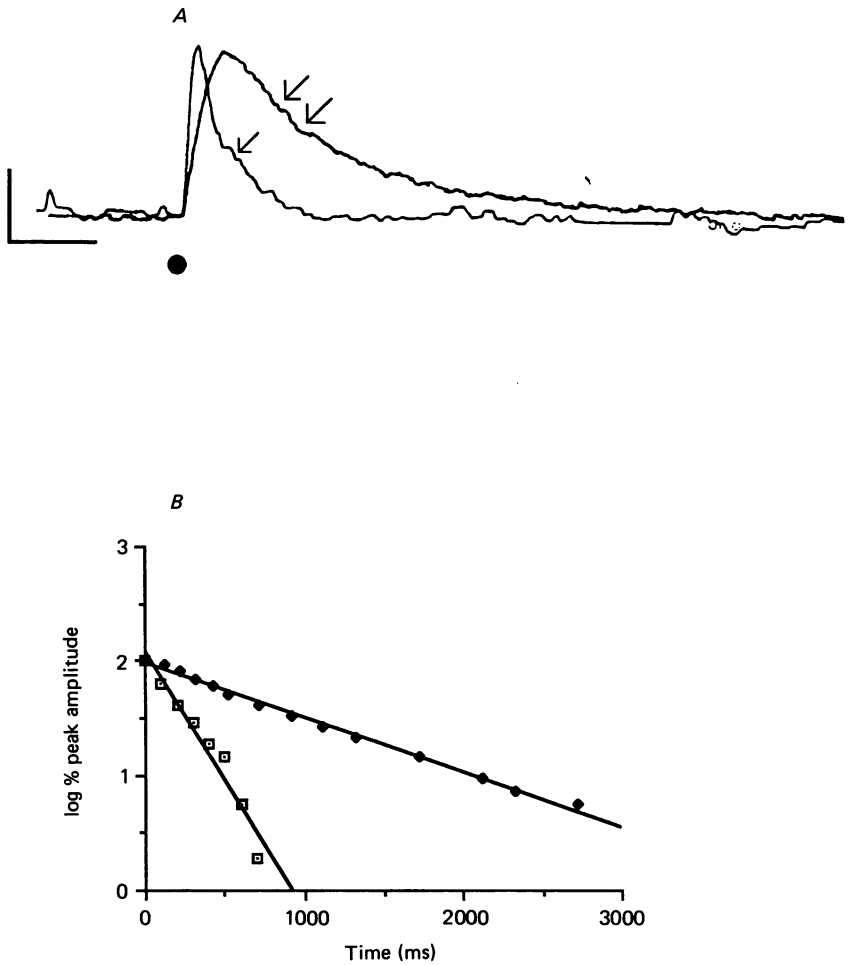

Fig. 5. Effect of temperature on ATP-evoked junction potentials in the guinea-pig vas deferens. *A*, ATP potentials evoked by local application (●) of the drug (10^{-4} M) at 35 and 25 °C. Pulse pressure and duration: 106 kPa, 12 ms. Drug-evoked junction potentials were prolonged at 25 °C (double arrows) as compared with control responses at 35 °C (single arrow). ATP potentials from different cells with similar resting membrane potentials (~ 64 mv) in the same tissue. Rise time and 50% decay time were as follows: 200 and 400 ms at 35 °C, 500 and 1400 ms at 25 °C. Calibration bars: 2 mV, 1 s. *B*, semilogarithmic plot of the decay phase of the ATP-evoked junciton potentials against time at 35 and 25 °C. ⊡: 35 °C ($r = 0.97$ for curve fit). ◆: 25 °C ($r = 1.00$ for curve fit). The slopes of the straight lines show that the exponential decay of the ATP response is greatly prolonged at the lower temperature.

TABLE 1. ATP potentials: effect of temperature

Temperature (°C)	Rise time (s)	50% decay time (s)	*n*
35	0·243 ± 0·05	0·663 ± 0·09	28
30	0·348 ± 0·09	0·895 ± 0·29	21
25	0·713 ± 0·36	1·960 ± 0·79	31

$P < 0.01$ in all cases.

EJPs. EJPs were almost completely abolished 30 min after the addition of α,β-methylene ATP to the superfusion fluid (Fig. 6*C*).

We have investigated the effect on the membrane potential of these cells of brief, local application of α,β-methylene ATP (Cunnane & Manchanda, 1987). Figure 7*A*

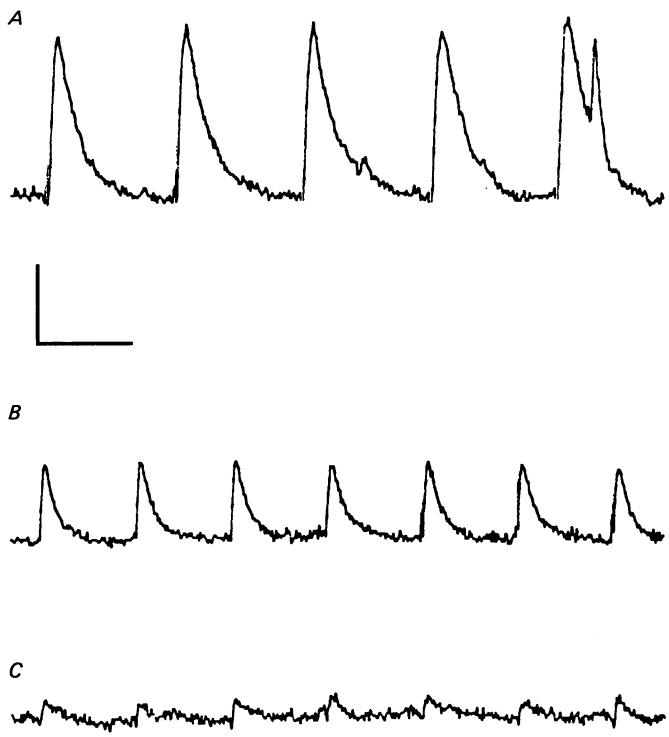

Fig. 6. Abolition of nerve-evoked EJPs in the presence of bath-applied α,β-methylene ATP (10^{-6} M). *A*, control; *B*, 20 min after the addition of α,β-methylene ATP to the superfusing solution; *C*, 30 min after addition of drug. Fully facilitated EJPs have been shown in each trace. EJPs were evoked at 1 Hz in the control trace (*A*) and at 0·7 Hz in the traces of *B* and *C*; the traces were obtained from three different cells but with comparable resting membrane potentials (65–70 mV). Calibration bars: 4 mV, 1 s for *A*; 4 mV, 1·5 s for *B* and *C*.

shows the membrane potential response to the application of a 6 ms pulse of 10^{-6} M-α,β-methylene ATP onto the surface of the tissue. A train of EJPs is shown preceding the α,β-methylene ATP-induced junction potential for comparison of the time courses. The drug produced a prolonged depolarization, the membrane potential returning to its resting level after 55 s. Such responses could be observed consistently and repeatedly, with the total duration of the response varying between 20 and 60 s. Some of this variation could be accounted for by noticeable drifts in membrane potential during the response to α,β-methylene ATP.

Cooling the preparation had no effect on the duration of the response to α,β-methylene ATP (Fig. 7*B* and *C*). If anything, responses at 25 °C were usually briefer

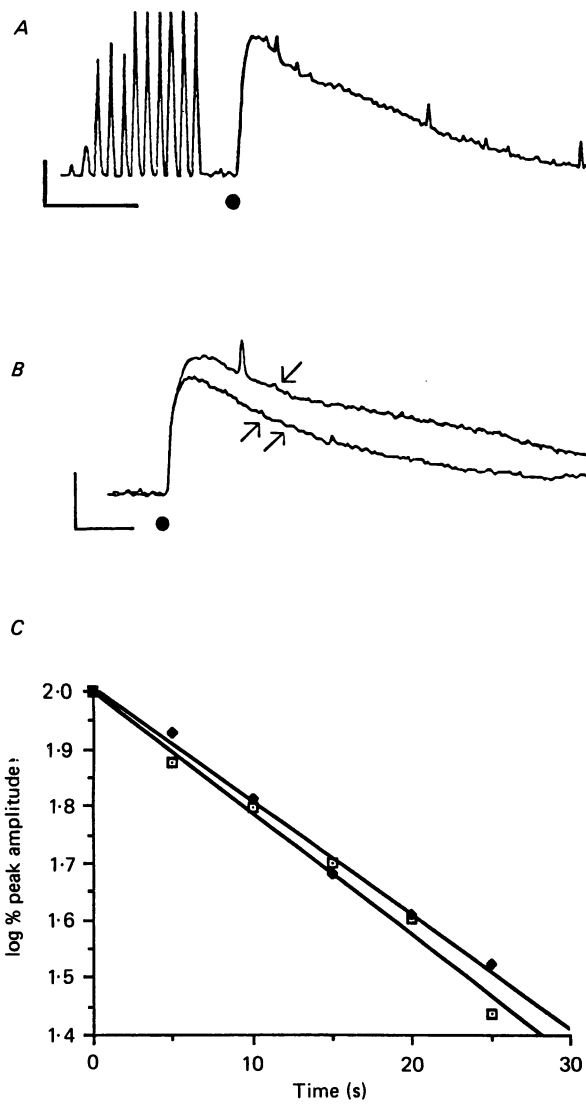

Fig. 7. Membrane potential responses to local application (●) of α,β-methylene ATP (10^{-6} M) at 35 °C (*A*) and at normal and cooled temperatures (*B*). The method of application of α,β-methylene ATP was identical to that for ATP to evoke membrane depolarizations. The drug was contained in a micropipette close to the recording electrode and applied to the surface by pressure ejection (106 kPa, 6 ms in all traces). Records obtained from different cells. *A* shows a train of EJPs preceding the α,β-methylene ATP response to facilitate comparison of the time courses. The random fluctuations in amplitude expected of fully facilitated EJPs were observed, but not recorded faithfully due to the limited frequency response of the plotter. Stimulation parameters for EJPs: 15 V, 0·3 ms. Resting membrane potential 65 mV. *B* shows that cooling did not prolong the drug-evoked response when the temperature of the preparation was lowered to 25 °C (double arrows) as compared with the response at 35 °C (single arrow). If anything, the decay time was somewhat shorter at the lower temperature. *C*, semilogarithmic plot of the decaying phases of α,β-methylene ATP responses against time at 35 °C (⊡) and 25 °C (◆). Calibration bars: *A*, 2 mV, 15 s; *B*, 2 mV, 10 s.

than the one illustrated. Decreasing the temperature of the preparation further, to 21 °C, also failed to alter the duration of the α,β-methylene ATP response. Mean duration of junction potentials evoked by α,β-methylene ATP were $46{\cdot}0 \pm 12{\cdot}1$ s, (mean $\pm$ S.D.) $n = 22$ at 35 °C and $43{\cdot}4 \pm 10{\cdot}6$ s, $n = 17$, at lower temperatures (21–25 °C). The difference was not significant ($P = 0{\cdot}49$, Student's t test). The lack of effect of cooling on α,β-methylene ATP-evoked responses is also shown in Fig. 7*C* which compares the exponential decay phases of responses at 35 and 25 °C in a semilogarithmic plot.

DISCUSSION

These results have a bearing upon the nature, and mechanism of inactivation, of the neurotransmitter mediating the EJPs in the guinea-pig vas deferens.

Sympathetic neurotransmitter mediating EJPs

Exogenously applied ATP can mimic the effects, both electrical and mechanical, of nerve stimulation on the smooth muscle cells of sympathetically innervated tissues (rodent vas deferens: Sneddon, Westfall & Fedan, 1982; Sneddon & Westfall, 1984; rat tail artery: Sneddon & Burnstock, 1984; rabbit ear artery: Suzuki, 1985). Our results in the guinea-pig vas deferens (Fig. 1) show that if care is taken to ensure unhindered access of exogenous ATP to the tissue surface, the electrical response to brief, local application of small amounts of ATP can remarkably faithfully mimic the shape and time course of the nerve-mediated EJP. This suggests, first, that the site of action, and the mechanism of action at this site, of endogenous transmitter and exogenous drug are likely to be identical; in the present study it would appear to be the action of ATP on postjunctional P_2-purinoceptors (Burnstock & Kennedy, 1986). Secondly, it indicates that the two substances share a common mechanism of inactivation at this junction, as the responses to both are terminated with equal rapidity. Our observations (Fig. 5) that ATP-induced depolarizations are prolonged on cooling in parallel with the effects of low temperature on EJPs, strengthens these contentions.

Since NA applied locally on its own did not elicit any junction potentials, and when applied together with ATP from the same micropipette at a ratio of 50 NA: 1 ATP failed to produce any noticeable change in the ATP-evoked junction potential, ATP alone appears to have an effect on the measurable electrical properties of the postjunctional smooth muscle cells, although NA and ATP are co-released from the sympathetic nerves innervating the vas deferens and mediate distinct contractile responses (see Burnstock, 1986).

Mechanism of inactivation of sympathetic neurotransmitter mediating EJPs

The major factor determining the lifetime of transmitter at sympathetic neuroeffector junctions was believed to be neuronal and extraneuronal re-uptake (of NA; Iversen, 1967). At sites where NA has been unequivocally shown to mediate synaptic potentials, blockade of NA uptake does markedly prolong these potentials (Suprenant & Williams, 1987). In the rodent vas deferens, however, Bell (1967) and Bennett & Middleton (1975) reported that noradrenergic re-uptake blockers, e.g.

cocaine and normetanephrine, had no consistent or significant effect on the time course of the EJPs.

An enzymatic inactivation of a non-noradrenergic transmitter mediating the EJPs in the guinea-pig vas deferens was postulated (Blakeley & Cunnane, 1982) on the evidence that EJPs are significantly altered (Kuriyama, 1964; Blakeley & Cunnane, 1982) – and in particular prolonged – on cooling the tissue. A temperature-sensitive, enzymatic mechanism of inactivation would be consistent with the proposed purinergic mediation of the EJPs in this tissue (Burnstock, 1986; Sneddon & Westfall, 1984; present results), as purines are normally hydrolysed enzymatically, and would explain the prolongation of ATP responses on cooling. Interference with transmitter inactivation mechanisms is known to prolong postjunctional responses to both endogenously released, as well as exogenously applied, transmitter at other neuromuscular junctions (Kuffler & Yoshikami, 1975*a*, *b*).

The prolongation of EJPs and ATP responses on cooling could arise from altered passive properties of the smooth muscle cell membranes, which largely determine the time course of decay of the EJP in this tissue at normal temperatures (Bywater & Taylor, 1980; Brock & Cunnane, 1987). However, our observation that the junctional currents underlying the intracellularly recorded EJPs (Brock & Cunnane, 1987) are prolonged on cooling (Fig. 4), indicates that an increased lifetime and duration of action of the transmitter also contributes to the prolongation of the EJPs at low temperature.

That the duration of action of the purinergic transmitter may be determined by enzymatic inactivation is further suggested by the nature of the electrical response to locally applied α,β-methylene ATP, the non-degradable analogue of ATP which is known to act on postjunctional P_2-purinoceptors to activate and subsequently desensitize them (Kasakov & Burnstock, 1983). α,β-Methylene ATP applied locally evoked membrane potential responses of comparable amplitude to ATP-evoked responses at a concentration (10^{-6} M) 100-fold lower than that of ATP (10^{-4} M), and the duration of the responses was 50–100 times greater than those to ATP. Both differences could be attributed to the resistance of α,β-methylene ATP to enzymatic degradation, resulting in an increased available concentration and duration of action of this compound at postjunctional purinergic receptors. Since α,β-methylene ATP is resistant to enzymatic degradation, its action would be independent of the activity of the enzyme. Thus variations in enzyme activity at different temperatures would not be expected to influence the time course of the responses to α,β-methylene ATP, as we have observed (Fig. 7*B*, and *C*).

The lack of effect of temperature on the responses to α,β-methylene ATP also indicates that the prolongation of EJPs and ATP responses on cooling is not likely to result from an effect of temperature on either the passive membrane properties of the smooth muscle cells, or the kinetics of the P_2-purinoceptor-activated channel mechanisms, as in either of these cases the duration of α,β-methylene ATP responses would also be expected to be altered upon cooling.

The identity of the enzyme(s) involved in the break-down of ATP and its analogues at these autonomic neuromuscular junctions remains to be established. Extracellular adenosine 5′-triphosphatases ('ecto' ATPases) may be good candidates for this function. ATPase enzyme activities distinct from the Na^+-K^+-activated,

Ca^{2+}-activated and mitochondrial ATPases have been demonstrated on the external surface of cell membranes in a variety of tissues (e.g. Hamlyn & Senior, 1983), and ATPase and 5′-nucleotidase have been localized in the cell membranes of the smooth muscle cells of the guinea-pig vas deferens (Forsman & Gustaffson, 1985). Although the role played by the enzymes at these extracellular sites is unknown, we suggest that one of their functions, at least in the guinea-pig vas deferens, may be the hydrolysis of ATP released as a neurotransmitter from the sympathetic nerves.

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