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PHARMACOLOGICAL EVIDENCE THAT ADENOSINE TRIPHOSPHATE AND NORADRENALINE ARE CO-TRANSMITTERS IN THE GUINEA-PIG VAS DEFERENS

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SUMMARY

1. The contractile response of the guinea-pig vas deferens to tetanic nerve stimulation was biphasic. The first phase was mimicked by exogenously applied ATP. The second more tonic phase was mimicked by exogenously applied noradrenaline (NA).

2. Intracellular micro-electrodes were used to record the electrical response of the vas deferens to nerve stimulation and to exogenously applied ATP and NA. Local application of ATP (10^{-5} to 10^{-3} M), by pressure ejection from a micropipette, produced a depolarization similar in magnitude and time course to the excitatory junction potential (e.j.p.). NA produced no such response.

3. Superfusion of the vas deferens with ATP and NA (10^{-6} to 10^{-4} M) produced a depolarization. The depolarization produced by NA was more gradual than that produced by the same concentration of ATP.

4. The ATP-receptor antagonist $ANAPP_3$ (arylazido aminopropionyl-ATP) preferentially antagonized the first component of the neurogenic contractile response and also antagonized the e.j.p.

5. The α-receptor antagonist prazosin preferentially antagonized the second phase of the neurogenic contractile response and enhanced the e.j.p. Similar results were obtained using the irreversible α-receptor antagonists phenoxybenzamine and dibenamine.

6. Cocaine (10^{-6} and 10^{-5} M) enhanced the second phase of the contractile response to nerve stimulation, but reduced the first phase. Lidocaine (10^{-5} and 10^{-4} M) had no such effect. Cocaine (10^{-6} and 10^{-5} M) reduced the magnitude of e.j.p.s at all stimulation frequencies from 1 to 8 Hz.

7. In the presence of the selective α_2-receptor antagonist yohimbine (10^{-7} M), both phases of the contractile response to nerve stimulation were enhanced to the same degree. This concentration of yohimbine also increased the magnitude of e.j.p.s. In the presence of 10^{-7} M-yohimbine, cocaine (10^{-6} and 10^{-5} M) still enhanced the second

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phase of the contractile response, but no longer reduced the initial phase of the contraction or e.j.p.s to the same degree.

8. In vas deferens from animals pre-treated with reserpine (2 mg/kg.day), the second phase of the contractile response to nerve stimulation was reduced but neither the first phase of the contraction nor the e.j.p.s was blocked.

9. These results suggest that the first phase of the neurogenic contractile response of the vas deferens and the e.j.p. are mediated by ATP acting on P_2-purinoreceptors, whereas NA mediates phase two, via α_1-adrenoceptors. The results also suggest that release of ATP and NA is influenced by a negative feed-back mechanism involving presynaptic α_2-adrenoceptors.

INTRODUCTION

The traditional view, often referred to as Dale's principle, that a nerve releases only one transmitter substance, has recently been challenged by many reports claiming that various nerves release more than one transmitter, although the evidence in many cases is equivocal (for a review see Burnstock, 1976). The biphasic nature of the contractile response of the guinea-pig vas deferens to nerve stimulation may be due to the release of two transmitters (Swedin, 1971; Ambache & Zar, 1971). The first component of the response is blocked by the ATP-receptor antagonist $ANAPP_3$ (arylazido aminopropionyl-ATP) and the second phase by α_1-receptor antagonists such as prazosin (Fedan, Hogaboom, O'Donnell, Colby & Westfall, 1981). The excitatory junction potential (e.j.p.) is also blocked by $ANAPP_3$ but not by α-receptor antagonists (Sneddon, Westfall & Fedan, 1982*b*). It has therefore been proposed that ATP and noradrenaline (NA) are released from sympathetic nerves in this tissue, and act as co-transmitters (Fedan *et al.* 1981).

We have now investigated the electrical events produced by motor nerve stimulation, the e.j.p. and the action potential, and the electrical and mechanical response of the muscle to exogenously applied ATP and NA. We have also tested the predictions of the co-transmitter hypothesis by examining the effects of cocaine, prazosin, yohimbine, dibenamine, phenoxybenzamine and reserpine on e.j.p.s and contractile responses evoked by trains of stimuli in the guinea-pig vas deferens. The results are fully consistent with ATP and NA being released as co-transmitters in this tissue.

Based on the results of these experiments, we discuss the possibility that ATP is the primary transmitter for initiation of e.j.p.s and subsequent firing of action potentials, while the contraction produced by NA is not dependent on the summation of e.j.p.s to fire action potentials. (A brief report of some of these results has already been published (Sneddon, Westfall & Fedan, 1982*b*).)

METHODS

Albino male guinea-pigs, 300–500 g, were killed by a blow to the head and subsequently exsanguinated. For experiments on contractile responses, the whole vas deferens was removed and mounted in a jacketed organ bath at 37 °C and bubbled with 95% O_2, 5% CO_2 in a physiological salt solution of the following composition (mM): NaCl, 113; KCl, 4·8; $CaCl_2$, 2·5; KH_2PO_4, 1·2;

$MgSO_4$, 1·2; $NaHCO_3$, 25; dextrose, 5·5. The bath had a volume of 1·5 ml and was continuously suffused at a rate of 3 ml/min. The vas deferens was passed through a pair of platinum ring electrodes. One end of the muscle was fixed and the other end attached to a Grass force-displacement transducer (FT 03) for monitoring tension. Contractile activity was displayed on a Grass polygraph. Field stimulation of the tissue was produced via pulses (0·5 ms, supramaximal voltage) from a Grass S 9 stimulator. In all experiments described below $ANAPP_3$ (10^{-4} M) was added to the bath containing the tissue and irradiated with a tungsten halogen projector lamp (DVY, 650 W, 3400 °K) for 20 min. The $ANAPP_3$ was then washed out for 10 min in drug-free solution. As described by Hogaboom, O'Donnell & Fedan (1980) $ANAPP_3$ becomes an irreversible antagonist of P_2-receptors in guinea-pig vas deferens when this procedure is used.

Intracellular micro-electrodes (20–60 MΩ filled with 3 M-KCl), were used to record e.j.p.s evoked by field stimulation using two platinum ring electrodes around the prostatic end of the vas deferens. Pulse width was 0·5 ms, at a supramaximal voltage. The signal from the micro-electrode was displayed on an oscilloscope (Tektronix) and recorded on magnetic tape, to be played back later on a pen-recorder. Current pulses could be injected through the micro-electrode. For experiments with $ANAPP_3$ on e.j.p.s, e.j.p.s were recorded from several cells, the vas deferens was then removed from the recording chamber and mounted in a continuously suffused organ bath for treatment with $ANAPP_3$, exactly as has been described for studying contractile responses. The tissue was then returned to the recording chamber and e.j.p.s recorded from several more cells after a period of at least 15 min wash-out of $ANAPP_3$ with fresh Krebs solution. Tissues treated with light for the same period without any $ANAPP_3$ served as 'light controls'. In all experiments recordings were made for only 60 min after returning the tissue to the recording chamber. ATP and NA could be locally applied to the tissue using a pressure-controlled micro-ejection device (General Valve Corp. Picospritzer II). The drugs were made up in Krebs solution, and a dye substance (Fast Green) added so that the spread of the drug could be viewed, using a Nomarski optics system. The micropipettes used to apply the drugs were ordinary micro-electrodes with their tips broken back. For experiments investigating the effect of local application of ATP and NA, two micropipettes were used in each experiment, one filled with ATP and the other with NA (with ascorbic acid added to prevent oxidation). The factors controlling the amount of each drug reaching the cell, the size of pipette (1–2 μm), distance of the pipette from the recording site, and pressure of the ejection were kept as similar as possible for the two drugs. Normally the effects of the two drugs were tested alternately, within seconds of each other. For these experiments the vas deferens was cut in a thin section 1–2 mm thick, about 1 cm long. The amount of drug applied was varied only by the duration of the pressure ejection (usually from 5 to 200 ms).

Tissues treated with dibenamine or phenoxybenzamine were placed in a solution containing the drug at 10^{-6} M for 30 min, and then washed in drug-free Krebs solution for a further 30 min before responses were obtained. Animals pre-treated with reserpine 2 mg/kg.day were injected intraperitoneally 48 and 24 h prior to the experiment.

Drugs

$ANAPP_3$ was synthesized by Drs J. P. O'Donnell and G. K. Hogaboom according to the method of Jeng & Guillory (1975). Prazosin HCl was a gift from N. Belcher, Pfizer Laboratories, Groton, CT. Reserpine (Sepasil) and other drugs were obtained commercially.

Statistics

All results on graphs show the mean ± S.E. of the mean. All mean e.j.p. sizes equal that of the fully facilitated e.j.p. Significance of the results was compared using a paired t test and considered to be statistically significant if $P < 0.05$.

RESULTS

Contractile responses to nerve stimulation, NA and ATP

The average time course of contractile responses of the guinea-pig vas deferens to nerve stimulation, exogenously applied ATP, β, γ-methylene ATP and NA are compared in Fig. 1. The frequency of stimulation and doses of agonist used were based

on previous results in which full dose–response curves were obtained. The magnitude of the stimuli used was chosen to be in the middle of the stimulus–response curve so that any reduction or enhancement would be obvious. The rapid, phasic contraction produced by ATP is similar in time course to the initial phasic portion of the neurogenic response. The response to NA is biphasic, with a very small, early

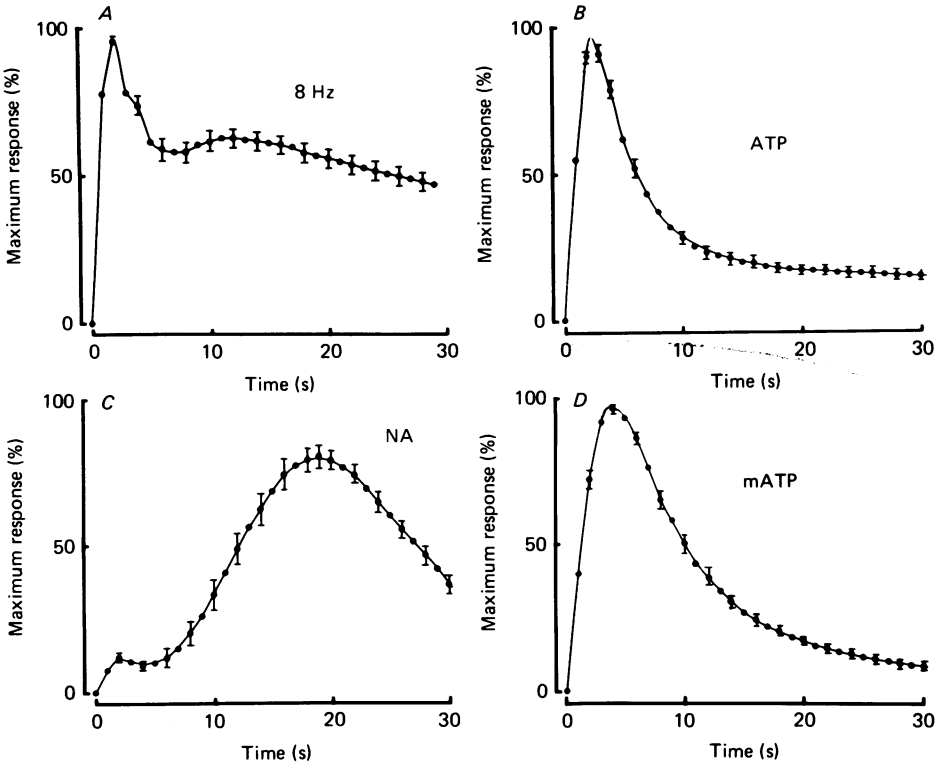

Fig. 1. The mean time course of the contractile responses of the guinea-pig vas deferens to: *A*, field stimulation at 8 Hz; *B*, 10^{-4} M-ATP; *C*, 10^{-4} M-NA and *D*, 10^{-5} M-β, γ, methylene ATP. All stimuli were applied for 30 s. Note the rapid transient response to the purines compared with the much slower response to NA. The response to nerve stimulation is biphasic. The data shown in this graph and similar ones that follow are the mean size of the contractile response of many muscles (n = 8–12), calculated at various times after application of the stimulus as a percentage of the maximum response which the tissue produced to that stimulus. The error bars in this and all subsequent graphs indicate the S.E. of the mean.

peak, similar in time course to the large peak produced by ATP, this then subsides before a much slower, more tonic contraction ensues, similar to the second phase of the neurogenic response.

Fig. 2 shows the results of similar experiments performed on tissues treated with $ANAPP_3$ (as described in the Methods). Here the first phase is antagonized, but the second is not affected. Fig. 3 shows the results of experiments after introduction of 10^{-6} M-prazosin. In this case only the second phase was antagonized. $ANAPP_3$ preferentially antagonized the response to exogenous ATP, and prazosin preferentially

antagonized the response to exogenous NA. While $ANAPP_3$ did not reduce the magnitude of the response to exogenously applied NA, it did slightly enhance the rate of rise of the response. Prazosin at 10^{-6} M completely abolished the response to exogenous NA and slightly depressed that to ATP (and, to a smaller degree, that to β,γ-methylene ATP), indicating that this dose is not entirely specific. Experiments

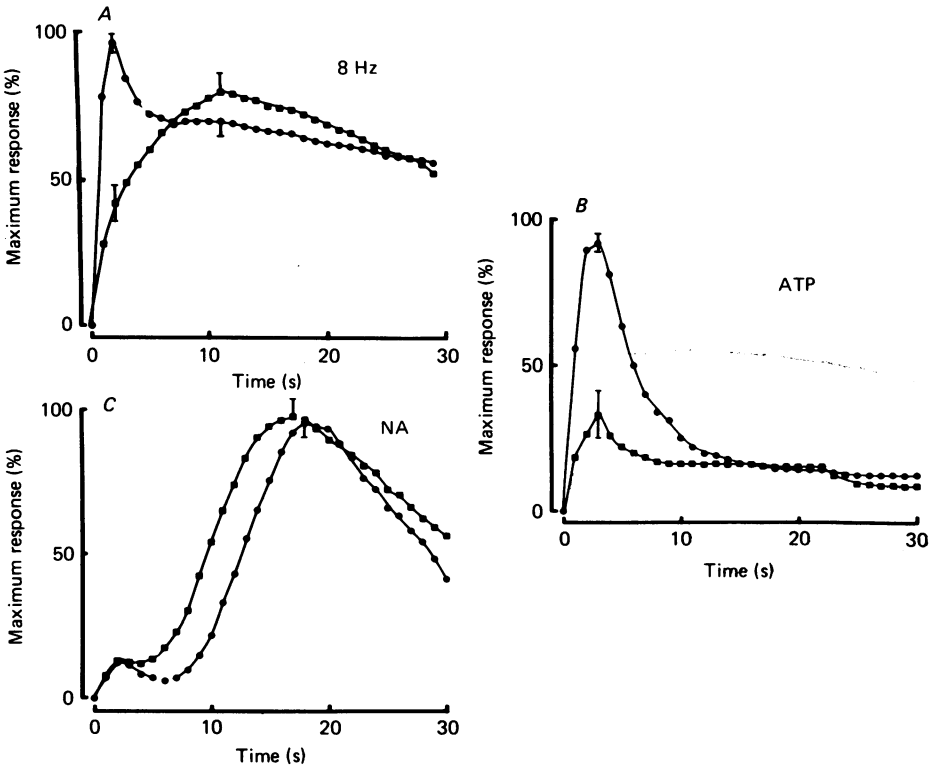

Fig. 2. The effects of $ANAPP_3$ (as described in Methods) on the contractile responses of the guinea-pig vas deferens to: *A*, field stimulation at 8 Hz; *B*, 10^{-4} M-ATP and *C*, 10^{-4} M-NA. $ANAPP_3$ produced a significant reduction in the mean amplitude of the first phase of the response to nerve stimulation and in the mean amplitude of the response to exogenously applied ATP 10^{-4} M (*B*). $ANAPP_3$ did not reduce the mean amplitude of the second phase or the response to nerve stimulation or the response to exogenously applied NA (*C*). Control, ●—●; $ANAPP_3$, ■—■.

with lower concentrations of prazosin (3×10^{-8} and 10^{-7} M) produced less reduction of responses to ATP while still antagonizing the second phase and responses to NA (data not shown). Similar results were obtained with the irreversible α-receptor antagonists dibenamine and phenoxybenzamine (not shown).

The time course of the contractile response of the muscle to β,γ-methylene ATP (which is less readily hydrolysed than ATP) was also examined. This compound was approximately 30–50 times more potent than ATP, but the time course of the contractile response was very similar to that produced by ATP, as were the effects of the other treatments described below.

The slow rate of rise of the response to exogenous NA did not seem to be due to

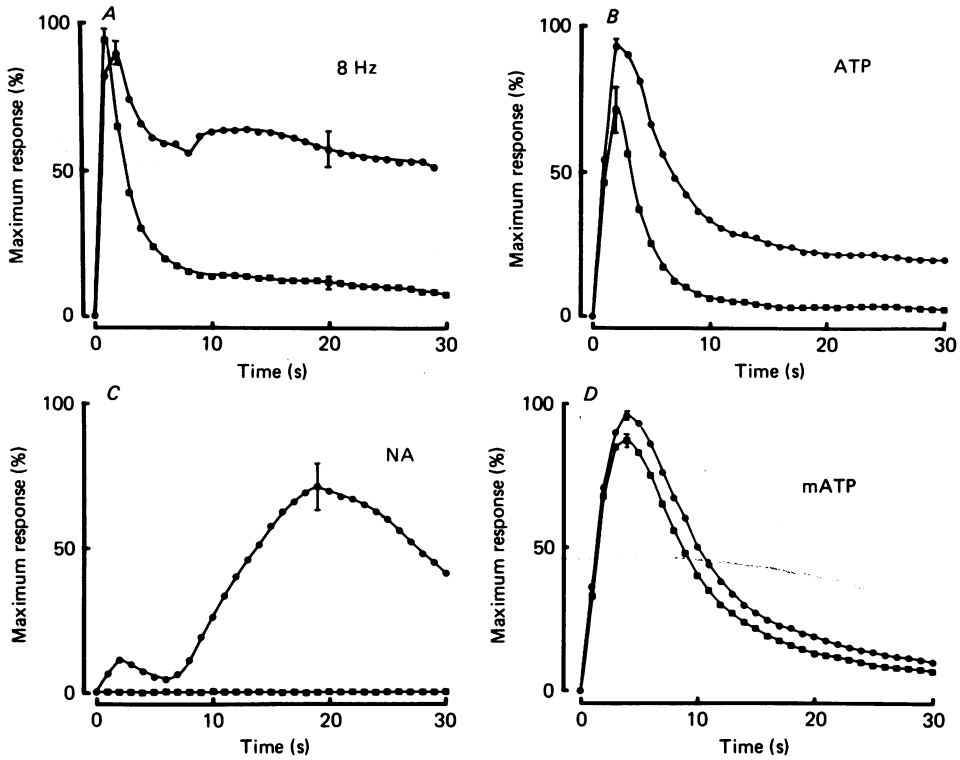

Fig. 3. The effect of prazosin 10^{-6} M on the contractile responses of the guinea-pig vas deferens to field stimulation at 8 Hz (*A*), to 10^{-4} M-ATP (*B*) to 10^{-4} M-NA (*C*) and 10^{-5} M-β, γ, methylene ATP (*D*). Prazosin significantly reduced the magnitude of the second phase of the response to nerve stimulation, but not the first phase. The response to ATP (*B*) and β, γ, methylene ATP (*D*) were reduced only slightly, whereas the responses to NA (*C*) were abolished. Control, ●—●; prazosin, ■—■.

slow diffusion of the drug through the muscle, since similar results were obtained when the muscle was split lengthwise to expose the lumen of the tissue to the drug (not shown).

Electrical responses to nerve stimulation, NA and ATP

Field stimulation of the motor nerves of the guinea-pig vas deferens produced e.j.p.s, which would summate to fire action potentials at a stimulation frequency of 1 or 2 Hz (Fig. 4). In tissues treated with $ANAPP_3$ (as described in the Methods section) e.j.p.s were greatly reduced in size, and even stimulation at 5–6 Hz often failed to produce an action potential (Fig. 5). The ability of $ANAPP_3$ treatment to reduce the average size of e.j.p.s is shown on the graph in Fig. 6. This Figure also shows that e.j.p.s recorded from muscles treated with light, but no $ANAPP_3$ were not reduced (see Methods), and that there is no difference between the resting membrane potential of control and treated cells.

Although $ANAPP_3$ greatly reduced the magnitude of the e.j.p.s, it did not appear

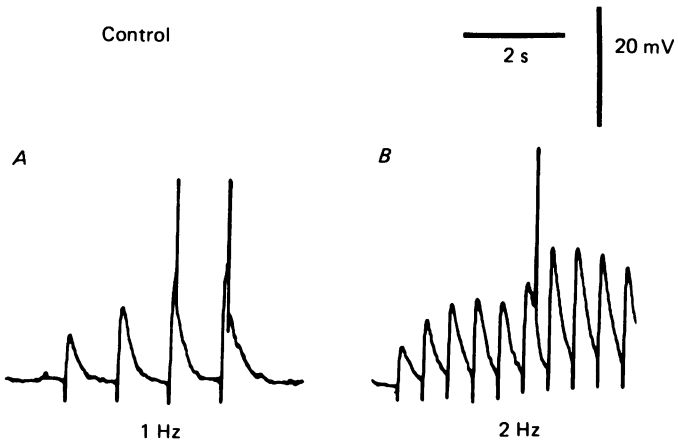

Fig. 4. *A* and *B*, recordings of control e.j.p.s from the vas deferens of the guinea-pig. In almost all cells recorded from, e.j.p.s summate to fire an action potential at 1 or 2 Hz, reaching threshold at about 15–20 mV below the resting membrane potential of the cell. The full magnitude of the action potential is not shown. The vertical deflexion preceding each e.j.p. is the stimulus artifact (on this and all successive traces). In *B*, the action potential has been propagated from a cell other than the one being recorded from.

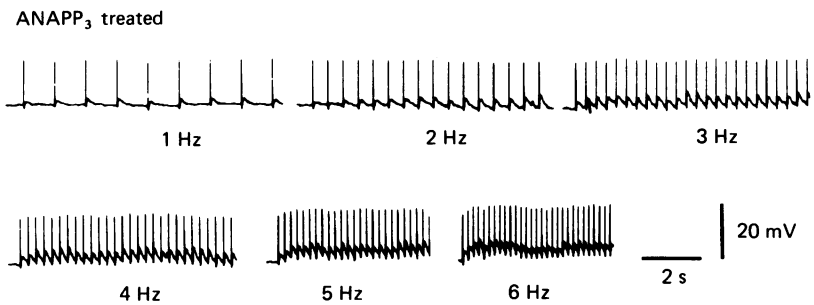

Fig. 5. E.j.p.s recorded from guinea-pig vas deferens treated with $ANAPP_3$, as described in the Methods. (The traces are sections of a continuous record from the same cell.) Note that e.j.p.s are very much reduced and, in this particular cell, even stimulation at 6 Hz did not evoke an action potential.

to change threshold, or the ability of the cell to fire an action potential. In all the experiments where e.j.p.s were recorded from $ANAPP_3$-treated cells, e.j.p.s never summated beyond the normal threshold without an action potential being fired, or the cell contracting and expelling the electrode (presumably due to action potentials being fired).

The competitive α_1-receptor antagonist prazosin (10^{-6} M) did not reduce the magnitude of e.j.p.s (top panel, Fig. 7). Fig. 7 also shows that during continuous recordings from single cells the average e.j.p. size increases with time after the introduction of 10^{-6} M-prazosin. The irreversible α-receptor antagonist phenoxybenzamine did not reduce e.j.p.s significantly, even at 10^{-6} M, which completely abolished the second phase of the contractile response.

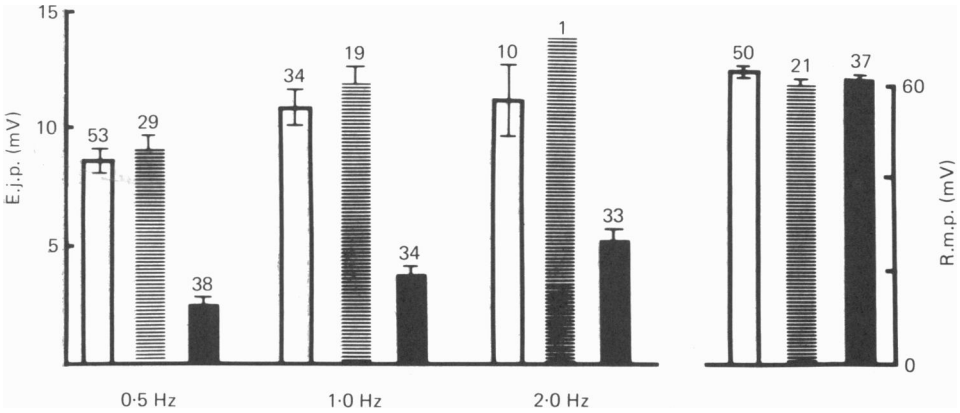

Fig. 6. The average size of the e.j.p.s recorded from control tissues (□); tissues treated with $ANAPP_3$ (■) and tissue treated with light in the absence of $ANAPP_3$ (▤) (as described in the Methods). At 0·5, 1·0 and 2·0 Hz there is no difference between control and light-treated muscles, but $ANAPP_3$-treated muscles had e.j.p.s which were considerably smaller. On the right-hand side is shown the average resting membrane potential of cells from the three groups, none of which is significantly different. The numbers above each column indicate the number of cells recorded from.

Local application of ATP and NA

Fig. 8*A* shows sections of continuous recordings from a single cell onto which ATP and NA were locally applied for short periods by use of a micro-pressure ejection device. The tissue responded to ATP with a rapid depolarization which, if the micropipette was close enough to the recording site, had a rise time similar to that of the e.j.p. (i.e. about 100 ms). However, NA applied in the same manner failed to produce depolarization. Application of ATP in amounts sufficient to depolarize the cell to reach threshold produced an action potential. Both ATP and NA when applied in this manner could produce a contraction of the muscle which could be seen through the microscope, and these contractions often resulted in the recording electrode being ejected from the cell. Fig. 8*B* shows sections of a continuous recording from a single cell of a muscle exposed to 10^{-5} M-cocaine, in which superfusion of ATP and NA for 20 s produced a depolarization. Even under these conditions, local application of NA was unable to depolarize the cell, whereas ATP produced a large, rapid depolarization. When hyperpolarizing current pulses (20 ms) were injected through the recording electrode (Fig. 8*A*) no change in the magnitude of the pulses was detected during drug application (see Discussion).

Superfusion of ATP and NA at various concentrations produced depolarization of the vas deferens. Fig. 9 shows sections of a continuous recording from a single cell. ATP and NA were approximately equipotent in producing depolarization, but the depolarization produced by NA was much more gradual than that produced by ATP. There was no change in the magnitude of the injected current pulses during the applications of either drug. 10^{-4} M-NA produced depolarization of the cell to threshold, and an action potential was fired before penetration of the cell was lost. The dose of ATP and NA required to produce a given degree of depolarization varied considerably from one cell to another.

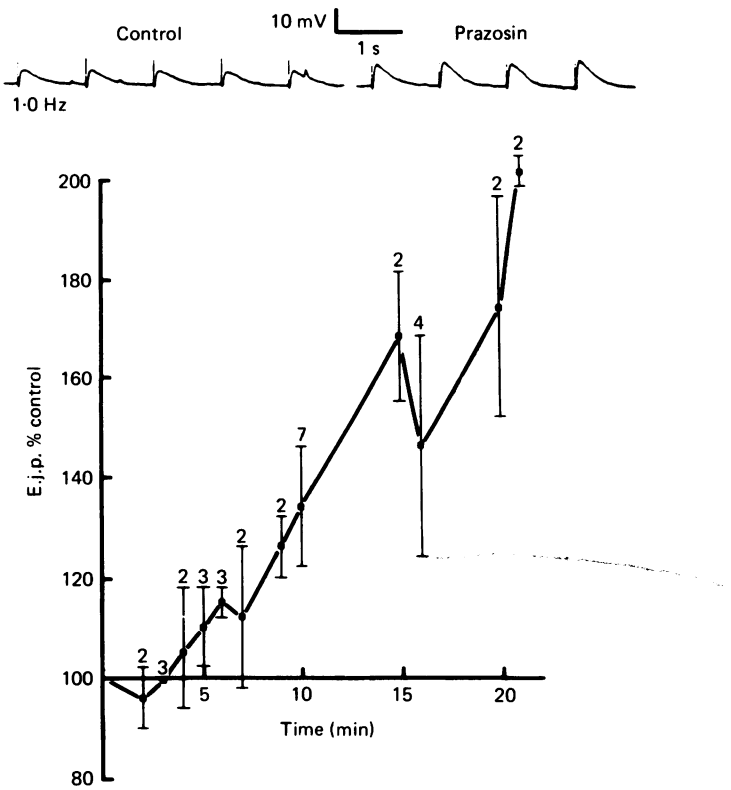

Fig. 7. The effect of prazosin on e.j.p.s in the guinea-pig vas deferens. Prazosin (10^{-6} M) did not reduce e.j.p.s in any of the cells recorded from, and usually increased e.j.p. size, as illustrated in the example at the top of the Figure. The average increase in e.j.p. size with time in the presence of prazosin is illustrated in the graph, which shows the relative change in e.j.p. size after introduction of prazosin during continuous recordings from the same cell. Stimulation was at 0·5 Hz. (The number of cells recorded from, n, is indicated by the number above each point.)

Cocaine and yohimbine

Exposure of the guinea-pig vas deferens to cocaine for short periods of time (10–15 min) produced effects on the contractile responses of the tissue which were reversible. The effects of cocaine were rapid in onset and very consistent. The first phase of the contraction was reduced and the second phase enhanced (Fig. 10). (In preliminary experiments the effect of cocaine (and other drugs) on the magnitude of the first and second phases of the responses was greatest in the middle of the frequency response curve, i.e. at about 8 Hz, therefore in subsequent experiments 8 Hz was usually used as the stimulation frequency.) These effects of cocaine were greater with 10^{-5} M-cocaine than with 10^{-6} M (Fig. 10). After returning to drug-free Krebs solution the response to nerve stimulation returned to control levels. Subsequent introduction of yohimbine (10^{-7} M) enhanced both phases of the response equally (i.e. by $58 \pm 8\%$ and $66 \pm 14\%$ respectively, $n = 6$). In the presence of yohimbine, cocaine still enhanced the second component of the contractile response, but no longer depressed

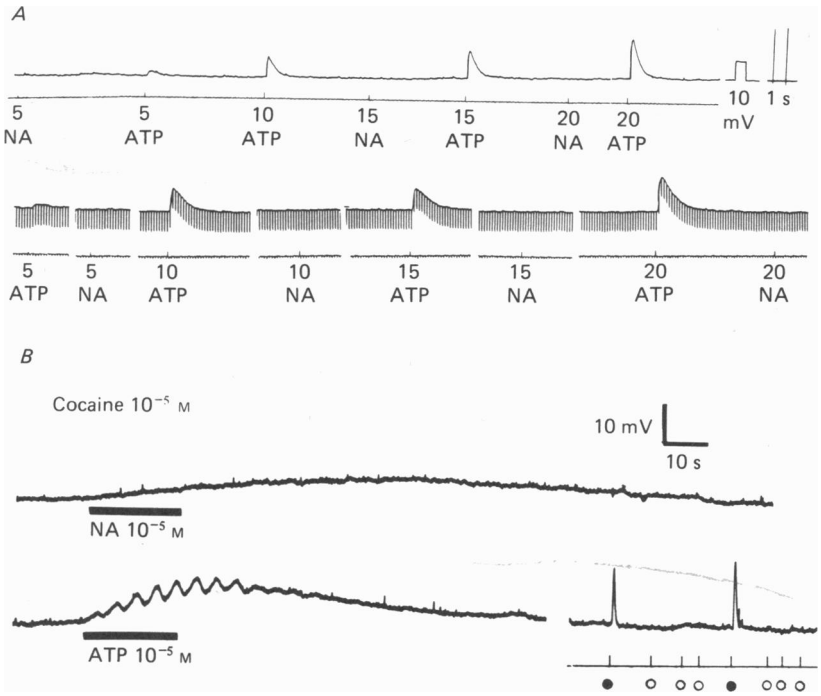

Fig. 8. *A*, two examples of the effect of local application of ATP and NA (10^{-4} M) on to the vas deferens on the membrane potential of a single cell. The numbers refer to the duration of drug application in milliseconds. ATP consistently produced a rapid depolarization which had a similar time course to an e.j.p. NA produced no depolarization. In the lower trace hyperpolarizing current pulses (20 ms) were applied, but no change in their magnitude could be observed during drug application (see Discussion). *B*, sections of a continuous recording of membrane potential from the same cell, in the continuous presence of 10^{-5} M-cocaine. ATP and NA produced depolarization of approximately equal magnitude when superfused on to the tissue for 20 s. ATP (●) also produced a large rapid depolarization when applied locally, but NA (○) did not. The oscillations in membrane potential produced by ATP superfusion in this cell were also seen in some other cells, but the more common response was that illustrated in Fig. 9.

the initial phase of the contraction. The effect of cocaine on the average size of the peaks of the two phases of the contractile response in the presence and absence of yohimbine is shown in Fig. 11. The data in Fig. 11 were obtained from experiments like that illustrated in Fig. 10. The ability of cocaine to depress the initial phase of the contractile response was not due to a local anaesthetic action, since if the experiment illustrated in Fig. 10 was repeated with 10^{-5} or 10^{-4} M-lidocaine, the contractile response was no different from that of the control, even after 30 min in lidocaine (not shown).

The effect of cocaine on e.j.p.s is illustrated in Fig. 12*A*. The effect of cocaine on the average size of e.j.p.s recorded from many cells in similar experiments are shown in Fig. 12*B*. Although the action of cocaine was variable in terms of its rate of onset, and the degree to which it reduced e.j.p.s (possibly related to the geometry of the recording sites), we never observed any enhancement of e.j.p.s in any of the cells

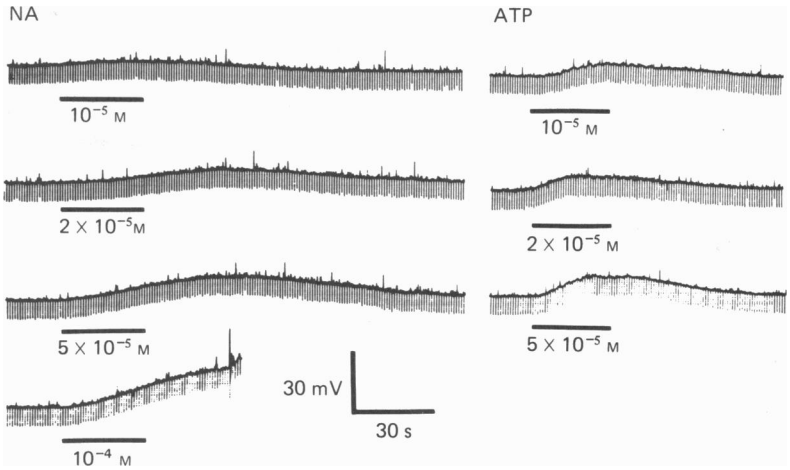

Fig. 9. Sections of a continuous recording from the same cell superfused alternately with ATP and NA for the period indicated by the bar (30 s). ATP and NA produced depolarization of similar magnitude, but that produced by NA was much more gradual. The downward deflexions are hyperpolarizing current pulses injected via the microelectrode, which did not change in magnitude during drug application (see Discussion). The upward deflexions are spontaneous e.j.p.s. In the final segment an action potential was recorded shortly before the electrode was ejected from the cell.

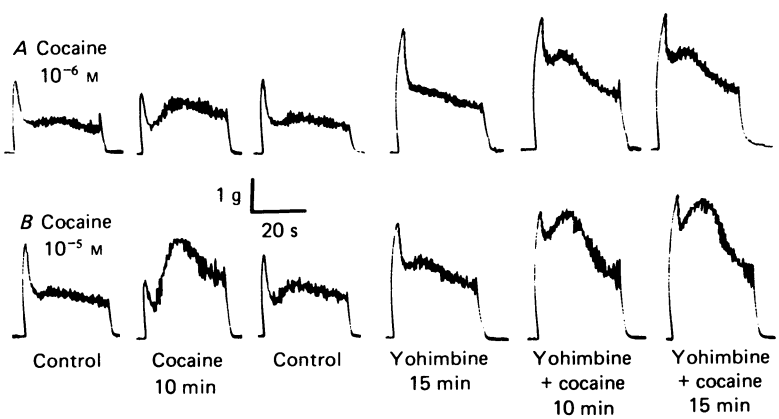

Fig. 10. Record of contractile responses of the guinea-pig vas deferens to field stimulation of the motor nerves at 8 Hz. The protocol of the experiments illustrated in *A* and *B* was identical except that in *A* the concentration of cocaine was 10^{-6} M, and in *B*, 10^{-5} M. Both *A* and *B* are sections of a continuous recording of tension from the same muscle. After 10 min in cocaine the first phase of the contraction was smaller, and the second phase larger than in the control. Introduction of 10^{-7} M-yohimbine enhanced both phases equally. Subsequent re-introduction of cocaine still enhanced the second phase of the contraction, but no longer reduced the first. These phenomena were also observed at stimulation frequencies of 4 and 6 Hz.

recorded from. In direct contrast to a previous report (Bell, 1967), we found that the frequency of stimulation required in order for e.j.p.s to summate to threshold and fire an action potential was much higher in the presence of cocaine (often 5–8 Hz) than in control cells (which usually fire at 1–2 Hz).

Yohimbine (10^{-7} M) caused a significant increase in the mean amplitude of the fully summated e.j.p. in a train. For example, at 0·5 Hz the mean amplitude of e.j.p.s from

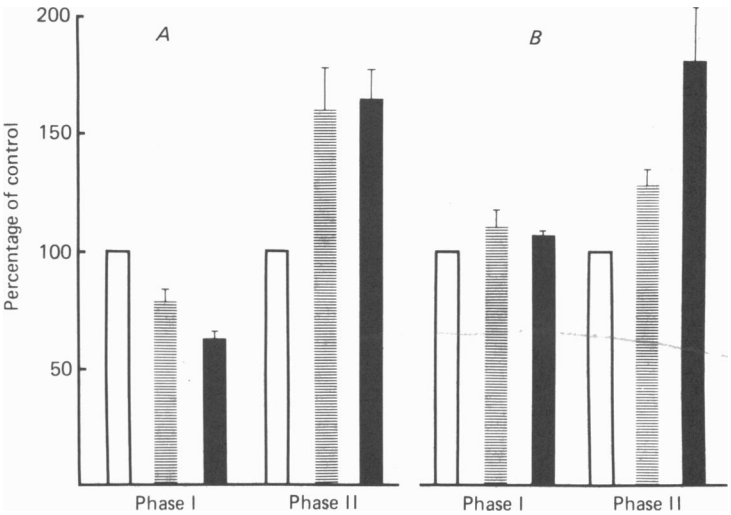

Fig. 11. Graph of the relative change produced by cocaine (as a percentage of control) in the mean size of the two phases of the contractile response of the guinea-pig vas deferens to nerve stimulation. Note that in the control (*A*) cocaine reduced the first phase of the response and enhanced the second phase, but in the presence of 10^{-7} M-yohimbine (*B*) the first phase is not reduced, although the second is still increased. These results were obtained from similar experiments to those illustrated in Fig. 10 ($n = 6$). Control (□); 10^{-6} M-cocaine (▤); 10^{-5} M-cocaine (■).

control cells was $13{\cdot}4 \pm 1{\cdot}1$ mV ($n = 18$) whereas in yohimbine-treated tissues e.j.p.s increased after 10 min to a mean size of $18{\cdot}9 \pm 1{\cdot}9$ mV ($n = 5$), i.e. an increase of about 45 %. This effect of yohimbine at the same low dose as altered the contractile responses is likely to be the result of the drug reducing negative feed-back regulation of transmitter release via presynaptic α_2-receptors. In the presence of 10^{-7} M-yohimbine, the reduction in the magnitude of e.j.p.s produced by cocaine was not as great as in its absence. This effect, however, was variable and only a few successful experiments could be performed since the addition of yohimbine potentiated e.j.p.s, so that even at 1 Hz or less threshold was often reached, producing contraction and loss of penetration of the cell by the micro-electrode. Since completed experiments could only be carried out on the few cells which did not reach threshold in the presence of yohimbine, this might bias the results obtained. Fig. 13*A* illustrates one of the few experiments in which e.j.p.s were measured after 10 min in the presence of yohimbine, and indicates that the addition of cocaine produced a smaller reduction in e.j.p. size than usual (compare Fig. 13*A* with Fig. 12*A*). The average reduction in e.j.p.s produced by 10^{-5} M-cocaine in the presence and absence of 10^{-7} M-yohimbine

is shown in Fig. 13*B*. Although the results are variable, the trend is consistent with the effect of cocaine observed in the experiments on contractile responses of the tissue.

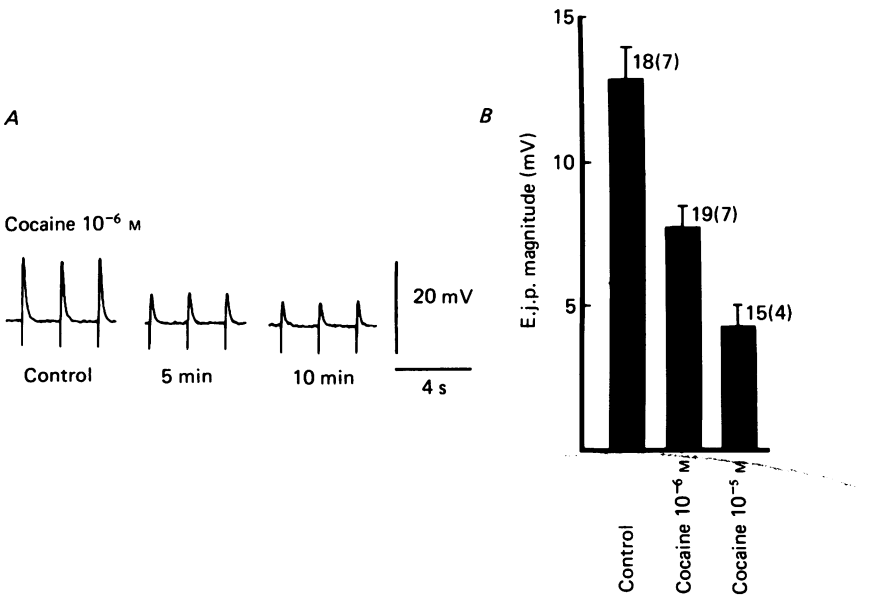

Fig. 12. *A*, the effect of cocaine (10^{-6} M) on e.j.p.s recorded from the guinea-pig vas deferens. Note that after the introduction of cocaine there was a progressive reduction in e.j.p. size. Notice also that cocaine did not alter the resting membrane potential of the cell. *B*, the effect of 10^{-6} and 10^{-5} M-cocaine on the mean amplitude of e.j.p.s recorded from the guinea-pig vas deferens. These values represent the mean amplitude of the fully summated e.j.p. in a train of e.j.p.s. The numbers beside the bars represent the number of cells recorded from, and the number in parentheses is the number of different tissues used.

Reserpine

The NA content of nerve endings in the guinea-pig vas deferens is reduced by 90 % if animals are pre-treated with a single dose of reserpine, 1 mg/kg, 24 h prior to the experiment (Westfall, Lee & Stitzel, 1975). The effect of treatment with reserpine (2 mg/kg for 2 days) on the time course of contractile responses to nerve stimulation, ATP and NA is shown in Fig. 14*A*. Reserpine clearly reduced the second but not the first phase of the neurogenic response. Responses to the exogenously applied agonists were not reduced by reserpine, but were in fact slighty enhanced, possibly due to the muscle developing supersensitivity to the transmitter after reserpine treatment (see Westfall, 1970). We found that this pre-treatment did not reduce the magnitude of summated e.j.p.s, e.g. at 1 Hz the mean size of e.j.p.s was $12{\cdot}5 \pm 0{\cdot}8$ mV ($n = 27$) which is not significantly different from e.j.p.s in control cells, $13{\cdot}0 \pm 1{\cdot}1$ mV ($n = 18$). An example of this result is shown on Fig. 14*B*.

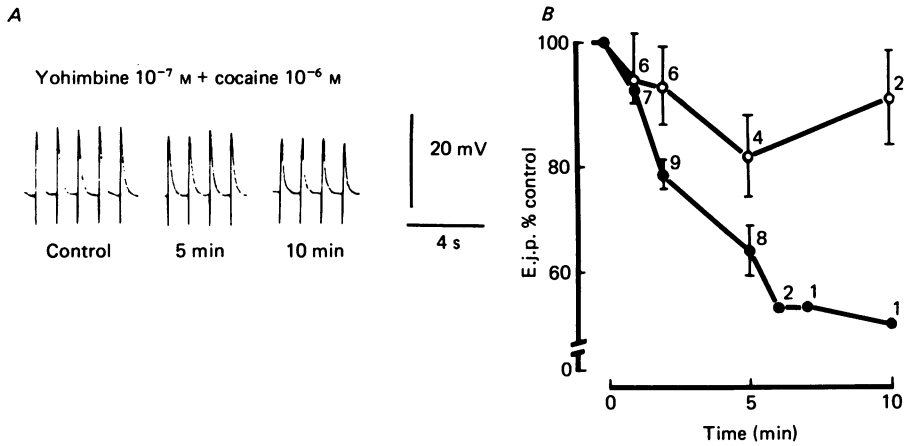

Fig. 13. *A*, sections of a continuous recording of e.j.p.s evoked in the guinea-pig vas deferens. All responses were obtained after 10^{-7} M-yohimbine had been present for over 15 min, so that the 'control' e.j.p.s shown on the left were already enhanced by about 45%. The addition of 10^{-6} M-cocaine produced only a slight reduction in e.j.p.s compared with that seen in the absence of yohimbine (see Fig. 12*A*). *B*, the effect of 10^{-6} M-cocaine on the mean amplitude of e.j.p.s recorded from the guinea-pig vas deferens. All data were obtained from continuous recordings of e.j.p.s from the same cell, so that the change in the magnitude of the e.j.p. with time in the cocaine-containing solution can be plotted as a percentage of the control e.j.p.s in the same cell. Before the introduction of cocaine, at time 0, the tissue was bathed either in a drug-free solution (●) or in a solution containing 10^{-7}M-yohimbine (○). Treatment with yohimbine increased e.j.p.s by an average of 45%. Although the results were variable, there is a trend which indicates that in the presence of yohimbine, cocaine's ability to reduce e.j.p. magnitude is less than usual. The number beside each point indicates the number of cells recorded from.

DISCUSSION

The smooth muscle of the guinea-pig vas deferens is densely innervated by sympathetic nerve fibres containing NA (Sjöstrand, 1965; Burnstock, 1970; Wakade & Kirpekar, 1971). The ability of prazosin to block the second component of the neurogenic response of the muscle indicates that the NA is released upon nerve stimulation and produces contraction via α_1-receptors. The time course of the contractile response to exogenously applied NA is consistent with it being responsible for the second phase of the neurogenic response. However, the initial phase of the contraction to nerve stimulation is resistant to prazosin and other adrenergic antagonists (see, e.g. Bentley & Smith, 1967; Ambache & Zar, 1971; Swedin, 1971). The first phase of the contraction is antagonized by the P_2-receptor antagonist $ANAPP_3$ (Fedan *et al.* 1981) and the time course of the contractile response of the muscle to exogenously applied ATP also indicates that ATP could mediate the initial phase of the contractile response via P_2-receptors.

The specificity of $ANAPP_3$ as a P_2-receptor antagonist has now been demonstrated in a variety of smooth muscles using a variety of agonists. In no instance has it been found that $ANAPP_3$ antagonizes responses to any agonist unrelated to ATP (Fedan *et al.* 1981; Westfall, Hogaboom, Colby, O'Donnell & Fedan, 1982; Sneddon, Westfall

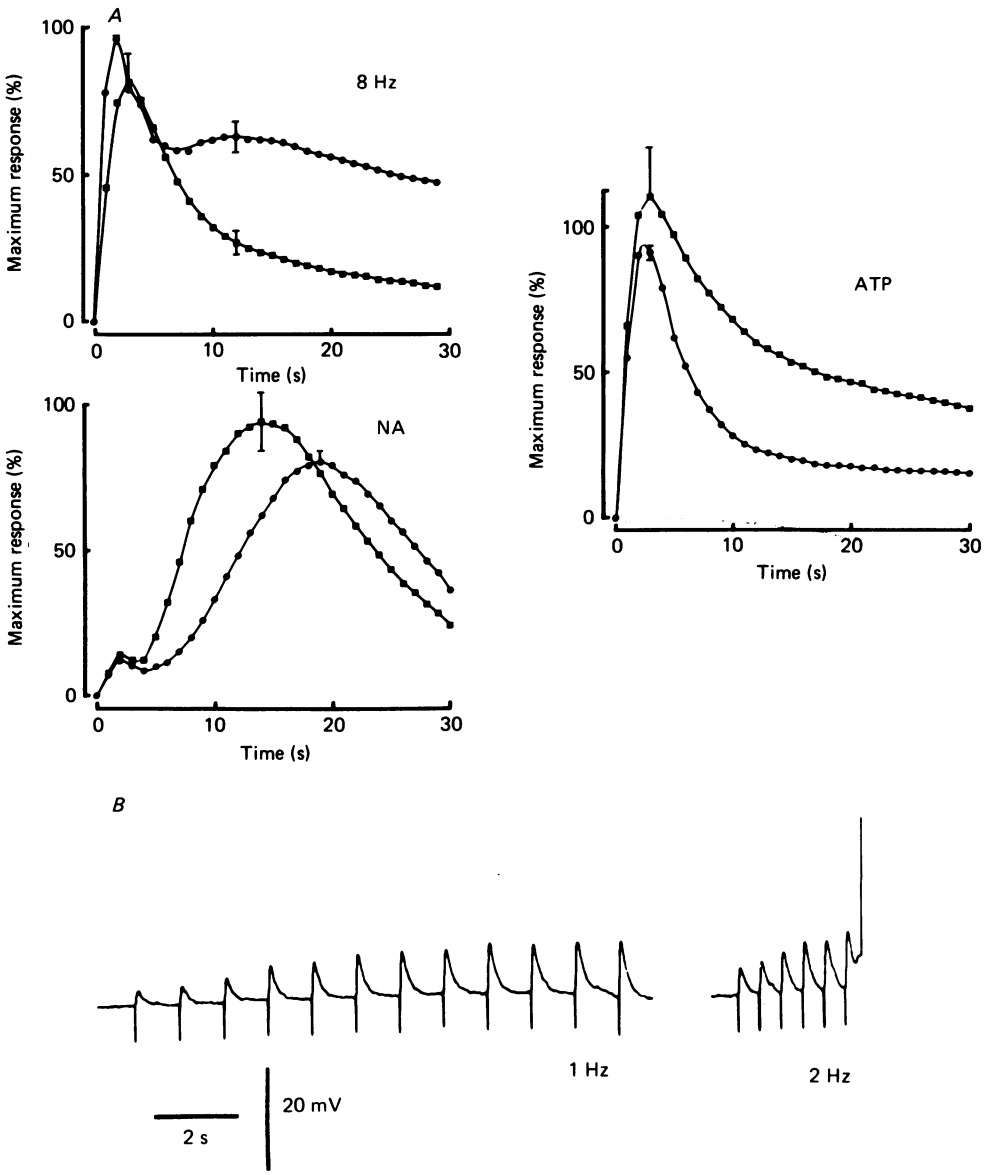

Fig. 14. *A*, the effect of reserpine pre-treatment on the time course of the contractile response of the vas deferens to nerve stimulation *A*, 8 Hz, *B*, ATP (10^{-4} M) and *C*, NA (10^{-4} M). The results are plotted as the mean response at various times as a percentage of the maximum response obtained in untreated muscles. Reserpine treatment clearly reduces the second phase to a much greater degree than the first. The responses to ATP and NA are enhanced, possibly due to the tissue developing supersensitivity ($n = 8$). *B*, the effect of reserpine pre-treatment (2 mg/kg, 24 and 48 h prior to the experiment) on e.j.p.s recorded from the guinea-pig vas deferens. E.j.p.s. at 1 Hz and 2 Hz are no different from control e.j.p.s in their final magnitude. Control, ●—●; reserpine, ■—■.

& Fedan, 1982*a*). Since $ANAPP_3$ treatment does not reduce the second phase of the contractile response, it is unlikely that there is any non-specific effect of the antagonist on the nerve or muscle in this preparation, i.e. it would seem that the amount of NA released and its post-synaptic effect are not changed.

In control tissues, electrical stimulation produces e.j.p.s which summate to fire an action potential. This seems to be the underlying mechanism by which the initial phase of the neurogenic contractile response is produced, since if nifedipine is used to block the action potential, the first phase is also inhibited (Blakely, Brown, Cunnane, French, McGrath & Scott, 1981). $ANAPP_3$ effectively antagonized the e.j.p.s in this muscle, and thus would be expected to reduce the number of cells reaching threshold to fire an action potential, explaining why $ANAPP_3$ reduces the first phase. Since prazosin, dibenamine and phenoxybenzamine did not antagonize the e.j.p. it seems unlikely that NA acting on α_1-receptors contributes to the e.j.p. in this preparation. The increase in e.j.p. magnitude during treatment with prazosin may be explained if prazosin at 10^{-6} M not only blocks post-junctional α_1-receptors but also, to some extent, prejunctional α_2-receptors, since this would inhibit the negative feed-back mechanism which normally reduces transmitter release during nerve stimulation.

If ATP is responsible for the e.j.p. then it should be mimicked by local application of the putative transmitter to the smooth muscle. The finding that ATP, but not NA, produced a depolarization of similar magnitude and time course to the e.j.p. is strong evidence in favour of the former being the mediator of e.j.p.s in this muscle. Even in the presence of 10^{-5} M-cocaine, locally applied NA could not produce a depolarization, suggesting that the lack of effect is not due to metabolism of the catecholamine before it can reach its site of action. It might be argued that locally applied NA does not produce depolarization because the receptors are discretely localized only in the tight junction between nerve and muscle, whereas the ATP can depolarize since its receptors are more widespread at extra-junctional regions. Although this argument cannot be dismissed, it none the less seems unlikely that NA could mediate e.j.p.s via α_1-receptors, since dibenamine and prazosin do not block e.j.p.s whereas $ANAPP_3$ does, and presumably all three antagonists have similar access to receptors, both at junctional and non-junctional regions.

The ability of NA and ATP to produce a depolarization when superfused for long periods indicates that both P_2 and α_1-receptors are able to change membrane permeability to one or more ions, even though no change in membrane conductance was observed. (This result may be explained in terms of the spread of injected current within the muscle, making it impossible to record true trans-membrane conductance changes, therefore no definite conclusion is drawn from these negative results.) In rat vas deferens the early phasic response to nerve stimulation and the response to exogenous ATP are blocked by nifedipine whereas the later phase and the response to NA are blocked by verapamil. This indicates that the different mechanisms of excitation–contraction coupling utilized by the two putative transmitters could correspond to those used by ATP and NA added exogenously (French & Scott, 1983).

From the above results we propose that neurotransmission in vas deferens is as outlined in Fig. 15, involving ATP and NA as co-transmitters. Some pharmacological tests of this hypothesis are discussed below.

Cocaine potentiates the contractile response of a variety of smooth muscles both to exogenously applied NA and to stimulation of adrenergic nerves (see, e.g. Iversen, 1965), and it is now generally accepted that this is due to an inhibition of neuronal uptake, reducing inactivation of the catecholamine (Trendelenburg, 1972).

In the guinea-pig vas deferens the contractile response of the muscle to nerve stimulation has previously been reported as being increased by cocaine

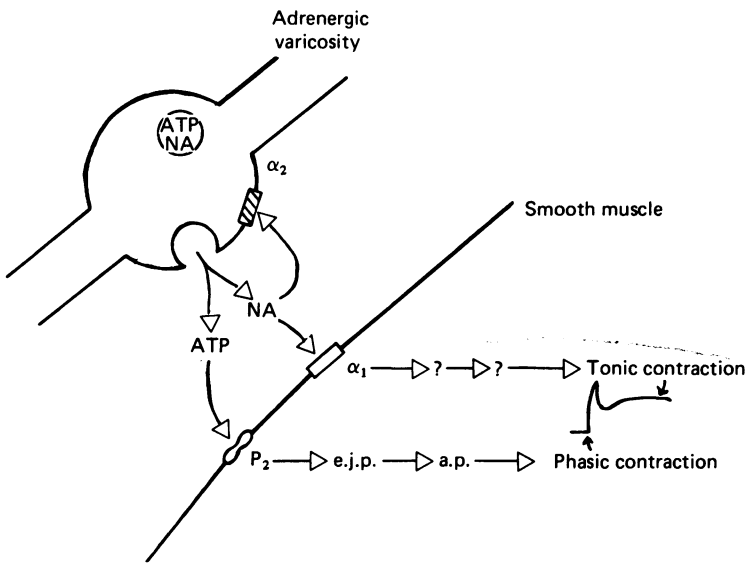

Fig. 15. Schematic representation of the co-transmitter hypothesis proposed for guinea-pig vas deferens. When the nerve varicosity is depolarized, it releases ATP and NA which act on P_2 and α_1-receptors respectively of the smooth muscle cell. The first phase of the contractile response results from the action of ATP depolarizing the cell and depends on the summation of e.j.p.s to fire action potentials (a.p.). The second phase is mediated by α_1-receptors by a mechanism which is independent of action potentials, and may be potential dependent or potential independent. ATP is rapidly inactivated to adenosine, which may regulate transmitter release by a presynaptic action on P_1-receptors. NA may also regulate release presynaptically via α_2-receptors.

(10^{-6} to 10^{-5} g/ml, Bell, 1967) or as being reduced by cocaine (5×10^{-8} to 5×10^{-4} M, Ambache, Dunk, Verney & Zar, 1972). In the present study we confirm both of these findings. If a short train of pulses is used, only the early component of the contractile response is observed, and this is reduced by cocaine. A longer train of pulses will show the second phase of the response which, being adrenergically mediated, will be enhanced by cocaine. A very important point demonstrated by Ambache *et al.* (1972) is that the inhibitory action of cocaine is not due to a local anaesthetic effect of the drug, since cocaine does not depress the twitch response in reserpine-treated animals (even at concentrations as high as 5×10^{-5} M). Our finding that lidocaine, a potent local anaesthetic, is unable to reduce the contraction even at 10^{-4} M also indicates that cocaine's effect is independent of any local anaesthetic action. The ability of cocaine to reduce the first phase of the contractile response can be explained if we assume that the two transmitters (ATP and NA) are released from the same nerve varicosity,

indeed from the same vesicle. In control tissues, the released NA inhibits further transmitter release by negative feed-back action on presynaptic α_2-receptors. Introduction of cocaine reduces the number of vesicles released by increasing the amount of NA activating these α_2-receptors. In reserpine-pre-treated animals the contractile response is largely monophasic, i.e. the adrenergic component of the response is lost, since little NA is released, and since there is now no negative feed-back control of transmitter release cocaine will not alter release, or the size of the twitch response. This hypothesis is confirmed by our results using the α_2-receptor antagonist yohimbine. 10^{-7} M-yohimbine enhanced the two phases of the contractile response to the same degree, indicating that both are under the influence of the same α_2-receptor-mediated, negative feed-back control mechanism. The potentiating effect of this concentration of yohimbine on e.j.p.s. confirms that neuronally released NA normally inhibits the amount of purine released to produce the membrane depolarization which gives rise to the e.j.p.s. When the negative feed-back mechanism is blocked, introduction of cocaine will still enhance the post-synaptic action of NA, but its presynaptic action of reducing transmitter release will no longer be apparent, explaining the ability of yohimbine to prevent the inhibitory action of cocaine on the phasic portion of the contraction.

We propose that ATP mediates the e.j.p. (see Sneddon, Westfall & Fedan, 1982*b*). It seems that the contractile response elicited by neuronally relesed NA is mediated by an action potential-independent mechanism. This proposal is supported by the finding that prazosin (10^{-6} M) will completely block the adrenergic (second) component of the contractile response to nerve stimulation, but enhance the e.j.p. (Sneddon, Westfall & Fedon, 1982*b*). Conversely, increasing the adrenergic component of the contractile response by 60% in the presence of 10^{-5} M-cocaine results in a decrease in e.j.p. magnitude. These results (and others) would support the idea that activation of α_1-receptors by neuronally released NA to produce contraction is not mediated by action potentials in the guinea-pig vas deferens, but that NA does feed back on presynaptic receptors to influence release, and therefore e.j.p. size. We have also confirmed previous findings (Swedin, 1971) that reserpine reduces the second but not the first phase of the contractile response, and does not block the e.j.p. (Burnstock, Hollman & Kuriyama, 1964). If reserpine acts in the nerve varicosity by depleting storage vesicles of NA, but leaves ATP content unaltered, then this result would be consistent with the co-transmitter hypothesis.

In arterioles of the guinea-pig, α_1-receptor antagonists, such as prazosin, can block the contractile response of the muscle to NA locally applied at 'extra-junctional' sites, but not the depolarization produced by NA locally applied at 'junctional' sites or e.j.p.s evoked by nerve stimulation. This result prompted Hirst & Neild (1980) to postulate the existence of a new class of adrenoceptors which they later designated γ-receptors. Furthermore, they speculated that in vas deferens, the portion of the contractile response which is not blocked by α_1-receptor antagonists could be due to the action of NA on γ-receptors, i.e. only one transmitter is involved, but its effects are mediated via two groups of receptors. In light of this proposed alternative explanation, we have set out below a comparison of the evidence for the two hypotheses in relation to the neurotransmission process in the vas deferens, with particular emphasis on the question 'are the e.j.p. and first phase of the contractile response mediated by NA acting on α-receptors or by ATP acting on P_2-receptors?'

1. Blockade of e.j.p.s and the first phase of contraction by ANAPP$_3$. If ANAPP$_3$ is selective for P$_2$-receptors this is strong evidence that e.j.p.s and the first phase of the contraction are ATP mediated, however ANAPP$_3$ may also block γ-receptors, although no evidence is available on this issue. There is no known γ-receptor antagonist, so the hypothesis that these receptors mediate e.j.p.s and the first phase of the contraction cannot be tested directly.

2. Mimicking of e.j.p.s and the first phase of the contraction. ATP exogenously applied to the vas deferens can mimic both e.j.p.s and the first phase of the contraction, both in magnitude and time course. NA applied in the same manner can mimic neither the e.j.p. nor the time course of the first phase of the contraction. In order to explain this finding in terms of the γ-receptor hypothesis, it might be claimed that the γ-receptors are located within 'tight junctions' between the nerve varicosity and muscle which are not accessible to exogenously applied NA, but are activated only by neuronally released transmitter. Again, this possibility is not amenable to direct investigation.

3. The action of cocaine. Cocaine depresses e.j.p.s and the first phase of the contraction, whereas it enhances the action of exogenously applied NA and the noradrenergically mediated second phase. The γ-receptor hypothesis could still be tenable if cocaine were a γ-receptor antagonist, for which there is at present no evidence in the guinea-pig vas deferens, and since locally applied NA does not produce a depolarization in the vas deferens, it is difficult to see how this possibility could be tested. The depolarization produced by superfusion of NA is not blocked by cocaine.

4. The action of reserpine. E.j.p.s and the first phase of the contraction are relatively resistant to reserpine pre-treatment, whereas the second phase of the contraction is considerably reduced. If NA mediates all three responses it would be likely that all three would be similarly reduced by depletion of the transmitter from the nerve varicosities. How this result could be explained in terms of the γ-receptor hypothesis is not known.

In view of the lack of evidence in support of the γ-receptor hypothesis in the vas deferens and the difficulty of testing it, as indicated above, we at present favour the more direct explanation of the results, i.e. that e.j.p.s and the first phase of the contraction are mediated by ATP and that the second phase of the contraction is mediated by NA.

The response of the vas deferens to a single pulse, rather than a train of pulses as used here, is also biphasic (McGrath, 1978); whether this is the result of the action of two transmitters is yet to be established.

In conclusion, we propose that the action of ANAPP$_3$, prazosin, dibenamine, phenoxybenzamine, cocaine, yohimbine, and reserpine on the contractile response of the guinea-pig vas deferens to stimulation of the motor nerves with a train of pulses can be best explained by the co-transmitter hypothesis.

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