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Sympathetic co-transmission to the cauda epididymis of the rat: characterization of postjunctional adrenoceptors and purinoceptors

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1 Electrical field stimulation (10 Hz, 60 V, 1 ms, for 10 s) produced monophasic contractions of isolated preparations of rat cauda epididymis which could be abolished by guanethidine, and attenuated by prazosin and α,β-methylene ATP.

2 The rank order of potency of adrenoceptor agonists in causing contraction of the preparation in the presence of the neuronal uptake blocker, nisoxetine (0.1 μM) was: adrenaline $\geqslant$ phenylephrine $\geqslant$ noradrenaline > clonidine > methoxamine > metaraminol > dopamine $\geqslant$ isoprenaline > xylazine.

3 Responses to the agonists were blocked by prazosin but not by propranolol or idazoxan.

4 The rank order of potency of purinoceptor agonists in causing contraction of the cauda epididymis was: α,β-methylene ATP > β,γ-methylene ATP $\geqslant$ 2-methylthioATP > ATP > ADP. AMP and adenosine did not cause contractions.

5 Contractile responses to the purine nucleotide analogues were blocked by repeated application of α,β-methylene ATP.

6 It is concluded that both ATP and noradrenaline may act as co-transmitters in the sympathetic nerves supplying the smooth muscle of the rat cauda epididymis, and that α_1-adrenoceptors and P_{2X}-purinoceptors are present postjunctionally.

Introduction

The epididymis is a single highly convoluted tube which transports sperm from the testis to the vas deferens. The last section, the cauda epididymis is an important site of sperm storage. It is continuous with the vas deferens and is the only section of the epididymis which contains innervated smooth muscle. It is innervated by the same branch of the hypogastric nerve that supplies the vas deferens (Mitchell, 1935).

It is well documented that the contractions of the rat vas deferens evoked by nerve stimulation are mediated through the release of noradrenaline and of adenosine 5'-triphosphate (ATP) which act post-junctionally at α_1-adrenoceptors (van Rossum, 1965; Leedham & Pennefather, 1986) and P_{2X}-purinoceptors (Burnstock & Kennedy, 1985) respectively. Little research has been carried out on neurotransmission to the cauda epididymis. There have been reports that α-adrenoceptors and muscarinic receptors mediating contractile effects are present on the smooth muscle (Laitinen & Talo, 1981; Pholpramool & Triphrom, 1984). Previous studies from this laboratory have also shown that isolated preparations of rat cauda epididymis contract in response to exogenously applied purine nucleotide analogues (Ventura *et al.*, 1990).

The present series of experiments was undertaken in order to characterize the excitatory adrenoceptors and purinoceptors present on the smooth muscle of the rat cauda epididymis and to investigate the possibility that as in the vas deferens, noradrenaline and ATP act as co-transmitters.

A preliminary report on the presence of excitatory postjunctional P_{2X}-purinoceptors has previously been presented (Ventura *et al.*, 1989a).

Methods

Isolated organ bath preparations

Male Long-Evans Hooded rats weighing 200–300 g were killed by cervical dislocation. The abdomen was opened and the cauda epididymides from each side of the animal were removed. With the aid of an Olympus S071 stereo dissecting microscope, the proximal part of each cauda epididymis (i.e. that closest to the vas deferens-cauda epididymis junction) was carefully unravelled and 4–6 cm sections were taken. Cauda epididymis sections were then cut into two 2–3 cm sections, giving four preparations from each animal.

In order to study the effects of adrenoceptor agonists and of nerve stimulation the sections were mounted in separate 30 ml organ baths. For studies of the effects of the purinoceptor agonists, 5 ml organ baths were used. Organ baths contained Krebs-Henseleit solution of the following composition (mM): NaCl 118.1, KCl 4.7, $MgSO_4$ 0.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7 and $CaCl_2$ 2.5. This solution was warmed to 35°C and bubbled with 5% CO_2 in 95% O_2. Preparations in 30 ml baths were attached by a thread at one end to a fixed platinum electrode in the tissue holder. The tissues mounted in 5 ml baths had one end of each section attached to small wire holders. The other end of each preparation was attached by another thread to an FT03 transducer and isometric contractions were displayed on a Grass polygraph (Model 79D). Tissues were positioned so that the longitudinal muscle fibres were oriented vertically under approximately 0.2 g resting force. These were allowed to equilibrate for 60 min, before the effects of agonists or of field stimulation were investigated.

Responses to field stimulation

Intramural nerve terminals were field stimulated by pulses applied from a Grass S88 stimulator via two platinum electrodes (10 mm apart) incorporated in the tissue holder. The stimulation parameters were 60 V and 1.0 ms duration at a frequency of 10 Hz for 10 s every 100 s. Antagonist drugs were added to the isolated organ baths once the contractile responses to field stimulation had stabilized. Following drug administration, when the electrically evoked contractions had again stabilized, the responses were measured and compared with those obtained before administration of the drug. The magnitude of contraction in the presence of antagonist drugs was then compared with the contraction remaining in control tissues over the same time course.

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Effects of agonists

Discrete concentration-response curves to agonists of unstimulated preparations of rat cauda epididymis were constructed. When the response to each concentration of an agonist had reached a plateau, the tissue was washed with three to five times the bath volume and was allowed 15 min to recover before the next concentration was applied. Only one concentration-response curve was obtained from each tissue and only one agonist was applied to any one tissue.

Inhibitors of uptake and antagonist drugs

Experiments conducted in the presence of α,β-methylene ATP; the adrenoceptor antagonists, prazosin, idazoxan or propranolol; or the inhibitors of neuronal and extraneuronal uptake of noradrenaline namely, nisoxetine or β-oestradiol, were carried out with the drug present in the bathing solution for the entire duration of the experiment.

Statistical analysis

Statistical analysis was performed by use of the standard formulae for calculating mean and s.e.mean. Student's non-paired t test was used to evaluate the significance of differences between mean values or, if more than two groups were compared one-way analysis of variance was used. In all cases, the criterion of statistical significance was taken as $P < 0.05$.

Mean log concentration-response curves were constructed by pooling data from individual log concentration-response curves constructed from tissues from 6 rats and linear regression analysis was undertaken to examine whether or not pairs of pooled lines were parallel, linear and coincident (Documenta Geigy, 1970).

Estimates of potency ratios for each agonist compared to noradrenaline or ATP were obtained by use of least squares regression lines fitted to the central portions obtained from each log concentration-response curve (Documenta Geigy, 1970).

Drugs

The following drugs were used: adenosine (Sigma); adenosine 5′ diphosphate (lithium salt) (ADP, Sigma); adenosine 5′ monophosphate (sodium salt) (AMP, Sigma); adenosine 5′ triphosphate (disodium salt) (ATP, Sigma); (−)-arterenol bitartrate (noradrenaline, Sigma); atropine sulphate (Sigma); clonidine hydrochloride (Boehringer Ingelheim); (−)-epinephrine bitartrate (adrenaline, ICN); guanethidine sulphate (Ciba-Geigy); 3-hydroxytyramine hydrochloride (dopamine, Sigma); idazoxan (Reckitt & Colman); (−)-isoproterenol bitartrate (isoprenaline, Sigma); metaraminol bitartrate (Merck, Sharp & Dhome); methoxamine hydrochloride (Burroughs Wellcome); α,β-methylene adenosine 5′ triphosphate (lithium salt) (α,β-methylene ATP, Sigma); β,γ-methylene adenosine 5′ triphosphate (sodium salt) (β,γ-methylene ATP, Sigma); 2-methylthioadenosine 5′ triphosphate (sodium salt · $4H_2O$) (2-methylthioATP, RBI); nisoxetine hydrochloride (Eli Lilly); β-oestradiol (Sigma); (−)-phenylephrine hydrochloride (Sigma); pirenzipine dihydrochloride (Boehringer); prazosin (Pfizer); propranolol hydrochloride (ICI); xylazine hydrochloride (Sigma).

Adrenaline, dopamine, isoprenaline, metaraminol, noradrenaline and phenylephrine were dissolved and diluted to required concentrations in a catecholamine diluent (mM: NaCl 154.0, NaH_2PO_4 1.2, ascorbic acid 0.2). Clonidine and xylazine were initially dissolved in 0.1 ml of 0.1 M HCl and then diluted to the required concentration in distilled water. β-Oestradiol was dissolved in ethanol. A stock solution of prazosin was made by initially dissolving it in glycerol and then making it up to volume with a 5% w/v dextrose solution (see Leedham & Pennefather, 1986). Dilutions to the required concentration were made with distilled water. All other drugs were dissolved and diluted to the required concentrations in distilled water.

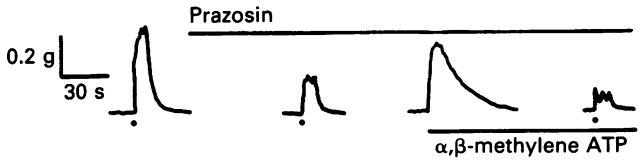

Figure 1 Typical trace showing the effects of prazosin (0.1 μM) and α,β-methylene ATP (10 μM) on responses to field stimulation (●) (10 Hz, 1 ms, 60 V, for 10 s every 100 s) of isolated preparations of the rat cauda epididymis. Note that application of α,β-methylene ATP caused a contraction of the tissue.

Results

Responses to field stimulation

Field stimulation (10 Hz, 1 ms, 60 V, for 10 s every 100 s) caused monophasic contractions of the isolated preparations of rat cauda epididymides (Figure 1). Responses were abolished by incubation of cauda epididymides in guanethidine (1 μM) ($n = 6$) (see Figure 2).

Prazosin and α,β-methylene ATP significantly attenuated the response to electrical nerve stimulation (Figure 1). Prazosin (0.1 μM) reduced the mean height of contraction to 45.0 ± 10.4% of control (Figure 2). Increasing the concentration of prazosin did not cause any further reduction in the size of the response. α,β-Methylene ATP (10 μM) reduced the height of the electrically evoked contractions to 63.9 ± 3.8% of control (Figure 2). The effects of these concentrations of the

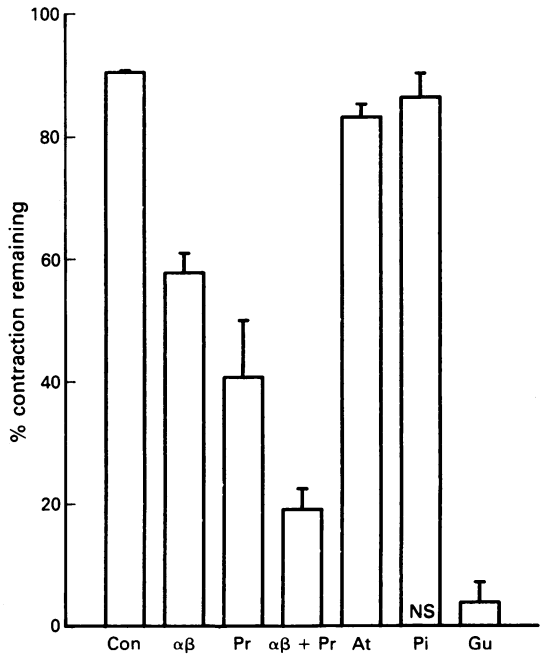

Figure 2 Mean percentage contractile responses remaining to field stimulation (10 Hz, 1 ms, 60 V, for 10 s every 100 s) following administration of: (Con) no drug, (αβ) α,β-methylene ATP (10 μM), (Pr) prazosin (0.1 μM), (αβ + Pr) α,β-methylene ATP (10 μM) and prazosin (0.1 μM), (At) atropine (1 μM), (Pi) pirenzipine (1 μM), (Gu) guanethidine (10 μM). Each column is the mean of estimates from 4–10 experiments and the vertical bars represent s.e.mean. Note that responses were significantly less than the drug-free time control for each drug treatment ($P < 0.05$, $n = 4$–10, unpaired t tests) except pirenzipine (Pi) ($P > 0.05$, $n = 4$, unpaired t test).

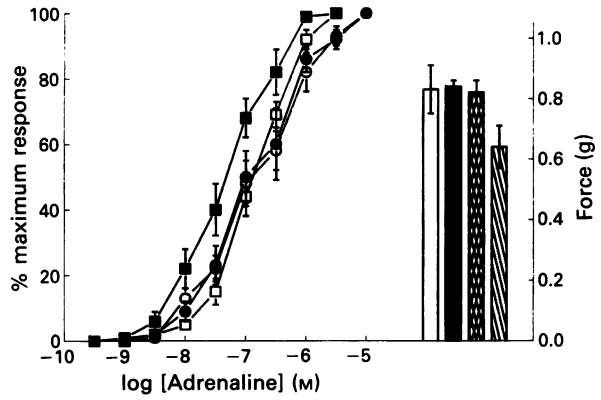

Figure 3 Mean log concentration-response curves for: adrenaline applied sequentially to isolated cauda epididymis segments (●) in control tissues, (○) in the presence of propranolol (1 μM), (■) in the presence of nisoxetine (0.1 μM) and (□) in the presence of β-oestradiol (10 μM). Points represent the means of estimates from six experiments and vertical bars represent the s.e.mean. The histogram columns represent the mean maximum force developed by (open) control tissues, (closed) in the presence of propranolol, (cross-hatched) in the presence of nisoxetine and (hatched) in the presence of β-oestradiol. Vertical bars represent s.e.mean.

two drugs were additive, such that in combination they reduced the mean size of contractions to 21.1 ± 3.8% of control (Figure 2).

The muscarinic receptor antagonist, atropine, caused a very small but significant attenuation of the electrically evoked contractions of the cauda epididymides. Thus atropine (1 μM) reduced the size of contraction to 91.9 ± 2.7% of control (Figure 2). Addition of atropine (1 μM), in the presence of both prazosin (0.1 μM) and α,β-methylene ATP (10 μM) did not cause any further reduction in the size of the response. The muscarinic M_1-receptor antagonist pirenzipine did not significantly reduce the size of contractions (Figure 2). Contractions in the presence of pirenzipine (1 μM) were 95.0 ± 4.3% of control.

Effects of inhibitors of uptake and propranolol on concentration-response curves to adrenaline

Adrenaline produced concentration-dependent tonic contractions of isolated preparations of the rat cauda epididymis. The mean negative log molar EC_{50} (i.e. the mean negative log molar concentration of agonist which gave a response which was 50% of the maximum response obtained to that agonist) in control preparations was 6.88 ± 0.12. Propranolol (1 μM) and β-oestradiol (10 μM) did not significantly affect the mean concentration-response curve to adrenaline; however nisoxetine (0.1 μM) shifted the adrenaline concentration-response curve significantly to the left (Figure 3) (potency ratio = 2.7, 95% confidence limits = 1.2–6.6, d.f. = 29). Maximum responses to adrenaline in the absence and in the presence of the various blocking drugs were not significantly different ($P > 0.05$, d.f. = 23, analysis of variance).

Adrenoceptor classification

Since nisoxetine but not β-oestradiol nor propranolol influenced responses to adrenaline, rank order of adrenoceptor agonist potency experiments were established in the presence of nisoxetine (0.1 μM) only. The catecholamines and all of the other sympathomimetic drugs tested produced concentration-related contractions of the cauda epididymides.

The mean maximum force developed in response to each agonist is shown in Table 1. Although analysis of variance showed a significant heterogeneity in the magnitudes of maximum responses to the agonists, none of the agonists produced mean maximal responses which differed significantly from that to noradrenaline ($P > 0.05$, $n = 6$ for all experiments, unpaired t tests).

Table 1 Mean negative log EC_{50} values, mean maximum force developed and potency ratios at α-adrenoceptors on the rat cauda epididymis[1]

Agonist	*−log EC_{50} values* (mean ± s.e.mean)	*Mean maximum tension developed* (g; mean ± s.e.mean)	*Potency ratio*
Adrenaline	7.32 ± 0.15	0.82 ± 0.04	1.6
Phenylephrine	7.17 ± 0.12	0.85 ± 0.04	1.1
Noradrenaline	7.12 ± 0.12	0.86 ± 0.08	1.0
Clonidine	6.61 ± 0.16	0.83 ± 0.07	0.31
Methoxamine	6.41 ± 0.12	0.81 ± 0.05	0.19
Metaraminol	5.94 ± 0.10	1.04 ± 0.04	0.07
Dopamine	5.06 ± 0.10	1.07 ± 0.07	0.009
Isoprenaline	4.97 ± 0.06	0.90 ± 0.04	0.007
Xylazine	4.88 ± 0.11	0.79 ± 0.06	0.006

[1] Nisoxetine (0.1 μM) present.
$n = 6$ animals for each experimental group.
Potency ratio = antilog ((neg log EC_{50} value for agonist) − (neg log EC_{50} value for noradrenaline)).

The mean log concentration-response curves for each of the agonists were parallel to that for noradrenaline. The mean negative log EC_{50} values determined from fitted regression lines and potencies relative to noradrenaline are shown in Table 1.

The order of potency for the nine adrenoceptor agonists was: adrenaline ≥ phenylephrine ≥ noradrenaline > clonidine > methoxamine > metaraminol > dopamine ≥ isoprenaline > xylazine.

Contractile responses evoked by methoxamine, isoprenaline and xylazine were antagonized by prazosin (0.1 μM); however, neither idazoxan (1 μM) nor propranolol (1 μM) ($n = 4$–5) inhibited these responses.

Purinoceptor classification

ATP, α,β-methylene ATP, β,γ-methylene ATP, 2-methylthioATP and ADP applied exogenously, each produced concentration-dependent, transient contractions of the rat cauda epididymides. AMP and adenosine were inactive. The potencies of ATP, β,γ-methylene ATP, 2-methylthioATP and ADP were low, and at 3 mM (the highest concentration of these agonists tested) maximum responses had not been attained (see Figure 4). α,β-Methylene ATP produced a maximal response at a concentration of approximately 30 μM. The mean log concentration-response curves for each of the active agonists were parallel to that for ATP (Figure 4).

The order of potency of these purines in producing contractions of the tissue was: α,β-methylene ATP > β,γ-methylene

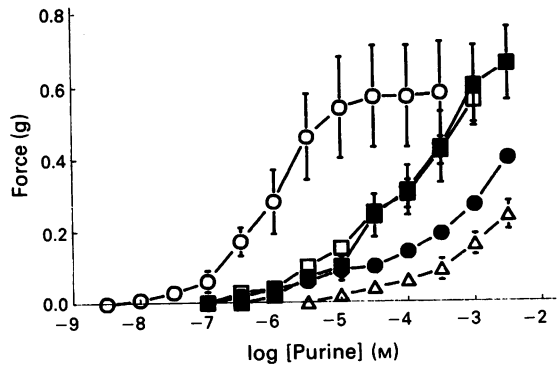

Figure 4 Mean log concentration-response curves for: (●) ATP, (○) α,β-methylene ATP, (■) β,γ-methylene ATP, (□) 2-methylthioATP and (△) ADP on control preparations of cauda epididymis. Points represent the means of estimates from six experiments. Vertical bars represent the s.e.mean.

ATP = 2-methylthioATP > ATP > ADP (Figure 4). ADP was 4.7 (95% confidence limits = 2.5–12.5, d.f. = 33) times less potent than ATP. α,β-Methylene ATP was 1150 (95% confidence limits = 483–2588, d.f. = 30) times more potent than ATP in producing contraction of the tissue. There was no significant difference in the potencies of β,γ-methylene ATP and 2-methylthioATP but these agonists were respectively 17.3 (95% confidence limits = 8.2–38.6, d.f. = 43) and 14.0 (95% confidence limits = 7.5–31.6, d.f. = 23) times more potent than ATP.

The contractions caused by these purine analogues could be almost totally abolished by desensitization of the receptor by prior exposure to α,β-methylene ATP (10 μM) ($n = 4$, for each agonist).

Discussion

The results of this study indicate that α_1-adrenoceptors and P_{2X}-purinoceptors mediating excitatory effects are present on the smooth muscle of the rat cauda epididymis, and that both noradrenaline and ATP may be released from sympathetic nerve terminals within the cauda epididymides in response to field stimulation.

The rank order of agonist potencies in causing contractions of preparations of rat cauda epididymides (i.e. adrenaline $\geqslant$ phenylephrine $\geqslant$ noradrenaline > clonidine > methoxamine > metaraminol > dopamine $\geqslant$ isoprenaline > xylazine) was substantially similar to that found in an earlier study from this laboratory using preparations of the epididymal segment of the vas deferens of this species (Leedham & Pennefather, 1986). The order of potency found in the present study also conforms to that considered by Wikberg (1978) to indicate the presence of α_1-adrenoceptors. In this investigation, as in our earlier study with vas deferens, phenylephrine was approximately three times more potent than clonidine. This estimate conforms to one of the criteria proposed by Starke in 1981 for subclassification of α-adrenoceptors as α_1. One difference, however, was that the potency of phenylephrine relative to that of noradrenaline was greater than expected. Thus it was found to be approximately equipotent with adrenaline and noradrenaline. Further evidence suggesting that the α-adrenoceptors were of the α_1-subtype has come from the observations that the effects of methoxamine and xylazine, which have high selectivity for α_1- and α_2-adrenoceptors respectively, were both blocked by prazosin but were unaffected by idazoxan. Evidence that isoprenaline was also acting at α_1-adrenoceptors in this preparation came from the fact that its effects, like those of methoxamine and xylazine, were blocked by prazosin, but not by either idazoxan or propranolol.

Since neither propranolol nor β-oestradiol enhanced the potency of adrenaline, and propranolol did not enhance the effects of isoprenaline, it can be assumed that postjunctional β-adrenoceptors and extraneuronal uptake play little or no functional role in this tissue. Despite the omission of propranolol in the present experiments, the EC_{50} obtained for isoprenaline in this study was similar to that obtained by Starke (1981) and Leedham & Pennefather (1986) both of whom included propranolol in their incubation media.

The rank order of potency of the naturally occurring purines i.e. ATP > ADP $\gg$ AMP, adenosine in producing contractions of the cauda epididymis is consistent with actions at P_2 rather than at P_1-purinoceptors (Burnstock, 1978). The relative order of potency of ATP and its methyl phosphate modified isosteres and 2-methylthioATP in causing contraction of the tissue, namely, α,β-methylene ATP $\gg$ β,γ-methylene ATP = 2-methylthioATP > ATP is broadly consistent with the subclassification of the P_2-purinoceptors mediating these effects as the P_{2X}-subtype (Burnstock & Kennedy, 1985). Indeed the relative potencies of these compounds on the cauda epididymis are very similar to those observed by Burnstock & Warland (1987), who compared the effects of these purines in causing contraction of preparations of mesenteric artery from rabbits. They found that α,β-Methylene ATP was over 1500 times more potent than ATP. β,γ-methylene ATP and 2-methylthioATP which were equipotent were approximately 30 times more potent than ATP.

The finding that the rank order of potency of ATP analogues obtained by Burnstock & Warland (1987) and that observed by us differs from that originally outlined by Burnstock & Kennedy (1985) may reflect 'between tissue' differences in the rate of catabolism of ATP. Until potent and selective inhibitors of ATP become available, P_2-purinoceptor subclassification based on agonist potencies alone will remain tentative. However, the relative agonist order of potency of the four agonists used in this study and in that of Burnstock & Warland (1987) on the rabbit mesenteric artery may prove to be a better description of the order of potency of purine nucleotide analogues at the P_{2X}-purinoceptor, since none of the studies reviewed by Burnstock & Kennedy (1985) actually employed all four of these agonists.

Further evidence for the classification of the purinoceptors in the cauda epididymis as being of the P_{2X}-subtype was the finding that responses to the agonists were blocked by prior application of α,β-methylene ATP. This acts selectively to desensitize P_{2X}-purinoceptors (Burnstock & Kennedy, 1985).

Investigations of the hypothesis that both noradrenaline and ATP are released from sympathetic nerve terminals within the rat vas deferens were prompted, in part, by observations that the response of this tissue to field stimulation are biphasic. The fast or 'purinergic' component of the response is prominent at the prostatic end of the tissue, whereas the slow or 'adrenergic' component of the response is more prominent at the epididymal end of the tissue (McGrath, 1978; Brown *et al.*, 1979; Rohde *et al.*, 1986). In the present study we have found that the contractions evoked by field stimulation of the cauda epididymis are monophasic and relatively slow. Nevertheless studies, using selective antagonists for α_1-adrenoceptors, P_2-purinoceptors and muscarinic cholinoceptors, were undertaken to deduce whether, as in the vas deferens, field stimulation of preparations of cauda epididymis led to release of substances other than noradrenaline from intrinsic sympathetic nerve terminals. Evidence that the field stimulation applied stimulated sympathetic nerve terminals was obtained in experiments using guanethidine which reduced stimulation-induced contractions by over 90%. Furthermore, although muscarinic agonists have been shown to cause contraction of preparations of cauda epididymides (Laitinen & Talo, 1981; Pholpramol & Triphrom, 1984; Ventura *et al.*, 1989b) neither pirenzipine nor atropine had any major effect on responses to field stimulation. In contrast, prazosin and α,β-methylene ATP both partially inhibited the contractile response to field stimulation, moreover, the effects of these two antagonists were additive. These findings taken together suggest that field stimulation of preparations of the rat cauda epididymis leads to the release of ATP as well as noradrenaline from sympathetic nerve terminals within the tissue.

The residual response to electrical stimulation in the presence of α,β-methylene ATP and prazosin may be due to direct smooth muscle stimulation, since there was also a small guanethidine-resistant residual response to electrical stimulation; or to the use of insufficient amounts of prazosin to block all of the postjunctional excitatory adrenoceptors present in the preparation. However, a third transmitter may be involved. Neuropeptide Y is perhaps the best candidate since it has previously been suggested that there is a dense innervation by neuropeptide Y-containing nerve fibres in the male reproductive tract of rodents. This peptide has been reported to induce a rise in resting tension of isolated preparations of mouse vas deferens by a postjunctional effect (Stjärne *et al.*, 1986).

In conclusion, the major findings of this study are that electrical stimulation of the nerve terminals present in prep-

arations of the cauda epididymis leads to contractions which can be inhibited by either α,β-methylene ATP or prazosin indicating that both ATP and noradrenaline act as cotransmitters. Secondly we have demonstrated that the smooth muscle of this tissue contains postjunctional α_1-adrenoceptors and P_{2X}-purinoceptors.

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