Br. J. Pharmac (1987), **92**, 349–355

Release of endogenous ATP during sympathetic nerve stimulation

[1]M.J. Lew & [2]T.D. White

Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

1 Vas deferens from guinea-pig was stimulated with a suction electrode and both contractions and release of endogenous ATP monitored

2 Release of ATP was tetrodotoxin-sensitive and increased when the number of stimuli was increased.

3 Release of ATP was not due to contraction of the muscle and persisted following block of contractions with prazosin and α,β-methylene ATP.

4 These results indicate that stimulation of the sympathetic nerves in the vas deferens releases endogenous ATP presynaptically, supporting a cotransmitter function for ATP with noradrenaline.

Introduction

The guinea-pig vas deferens responds to sympathetic nerve stimulation with a biphasic contraction (Ambache & Zar, 1971; Swedin, 1971). The second, slow onset contraction appears to be due to noradrenaline activating α-adrenoceptors on the smooth muscle (Ambache & Zar, 1971; Swedin, 1971). However, the first, fast phase of contraction, which is thought to be the result of summation of excitatory junction potentials (e.j.ps) and the generation of an action potential, does not seem to involve the activation of α-adrenoceptors in so far as it is not antagonized by α-antagonists (Ambache & Zar, 1971; Swedin, 1971; Fedan *et al.*, 1981; Sneddon *et al.*, 1982). There is mounting evidence that adenosine 5′-triphosphate (ATP), presumably co-released with noradrenaline from the sympathetic nerves, may act at specific P_2-purinoceptors on the smooth muscle of the vas deferens to produce the first phase of contraction (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984a; Stjarne & Astrand, 1984). However until now, evidence that endogenous ATP is indeed released during sympathetic nerve stimulation has been lacking. The release of ^{3}H-purines following transmural electrical stimulation of guinea-pig vas deferens preloaded with [^{3}H]-adenosine has been demonstrated (Westfall *et al.*, 1978), but it is not certain whether these ^{3}H-purines reflect released [^{3}H]-ATP or some other nucleotide or nucleoside, and also whether release occurred from the nerves or from the smooth muscle. In a previous attempt to detect the release of endogenous ATP from sympathetic nerves, transmural electrical stimulation of superfused, desheathed, guinea-pig vas deferens resulted in a release of endogenous ATP which was not reduced by the Na^+-channel blocker, tetrodotoxin (TTX), indicating that release was not the result of propagated action potentials in the nerves (White *et al.*, 1981). It is possible that both the nerves and muscle were directly depolarized by the stimulation conditions employed. In the present study, we determined whether ATP was released from sympathetic varicosities in the vas deferens when the hypogastric nerve and only a small portion of the smooth muscle were stimulated using a suction electrode on the prostatic end of the tissue. Our results demonstrate, for the first time, that electrical stimulation of sympathetic nerves in the guinea-pig vas deferens releases endogenous ATP presynaptically as a consequence of propagated action potentials in the nerves. This adds further weight to the hypothesis that ATP may function as a cotransmitter with noradrenaline in sympathetic nerves.

[1]Present address: Department of Physiology, University of Virginia, Charlottesville VA 22908, U.S.A.
[2]Author to whom all correspondence should be addressed at: Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7.

Methods

Protocol

Male guinea-pigs weighing 500–700 g were killed by cervical dislocation, exsanguinated, and their vasa

deferentia removed to Krebs solution gassed continuously with 95% O_2, 5% CO_2 at 25°C. The Krebs solution contained (mM): Na^+ 144, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HPO^{4-} 1.2, Cl^- 129, SO_4^{2-} 1.2, HCO^{3-} 25, glucose 11. The vas deferens (not desheathed) with silk thread attached to both the epididymal and prostatic ends was mounted in a 2 ml cylindrical, glass tissue bath and the prostatic end of the vas deferens, including the hypogastric nerve, gently sucked into a piece of polyethylene tubing which narrowed at the bottom of the bath. The electrodes consisted of a platinum wire ring at the base of the tissue and another platinum wire, enclosed within polyethylene tubing containing Krebs solution to complete the electrical circuit, located 1 cm below the tissue and ring electrode. An inlet tube at the bottom of the bath, to which was attached a length of polyethylene tubing and 3-way stopcock, permitted the introduction and removal of incubation media containing various drugs. Drugs were added in small volumes of stock solutions to incubation medium to achieve the desired final drug concentrations. Control medium was withdrawn from the bath and replaced with medium containing the appropriate drugs. The bath was attached directly against a photomultiplier tube (RCA 931B) and the assembly covered with reflective aluminium foil. The thread from the epididymal end of the vas deferens was attached to a Grass isometric force transducer, 1 g tension applied to the tissue and the preparation allowed to equilibrate for at least 30 min with changes of medium every 10 min. Then the entire system, including photomultiplier tube and force transducer, was covered with a box and shrouded with black felt to render the system light-tight. The tissue was usually stimulated at 20 Hz for 10 s with bipolar pulses of 150 V and 1 ms duration, through the two platinum electrodes, using a Grass S88 stimulator. To detect the release of ATP, the incubation medium was exchanged with medium containing 0.25 mg ml^{-1} firefly extract (Sigma FLE-50) and 0.1 mg ml^{-1} synthetic D-luciferin, prepared by adding 50 µl of 12.5 mg FLE-50 ml^{-1} H_2O (prepared fresh daily but allowed to stand at 25°C for 1 h before use) and 50 µl of 5 mg D-luciferin ml^{-1} H_2O (frozen stock kept on ice) to 2.5 ml of incubation medium. The light produced when the released ATP reacted with the luciferin-luciferase in the medium was detected by the photomultiplier tube connected to an Aminco microphotometer and this and the force of contraction of the vas deferens was recorded on a Grass polygraph. All experiments were conducted at least in triplicate and at 25°C because the luciferase is unstable at higher temperatures.

Drugs

ATP (disodium), α,β-methylene ATP (lithium), 4-aminopyridine, crude firefly extract (FLE-50), and D-luciferin was supplied by Sigma. Tetrodotoxin (TTX) was from Calbiochem, tetraethylammonium (TEA) from K & K Chemicals and prazosin from Pfizer.

Results

Previous studies have shown that 4-aminopyridine potentiates the contractile response to transmural stimulation and increases the release of noradrenaline in the vas deferens (Thesleff, 1980). In preliminary experiments, we found that inclusion of either 300 µM 4-aminopyridine or 1 mM tetraethylammonium in the medium increased the stimulation-evoked release of ATP at least 5 fold and produced a light signal of sufficient size and reproducibility to permit the subsequent systematic analysis of evoked ATP release. 4-Aminopyridine was included in all subsequent experiments. Figure 1 shows a typical record of the release of ATP produced when guinea-pig vas deferens was stimulated electrically with various numbers of pulses using a suction electrode as described in Methods. The contraction of vas deferens was accompanied by a release of ATP as indicated by the production of a light signal; moreover, release of ATP increased when the number of stimuli was increased. It is unlikely that the release of ATP was due to the contraction of the muscle *per se* because the addition of 100 µM phenylephrine to the bath, which contracted the tissue to a similar extent as electrical stimulation, failed to release ATP from the preparation (Figure 2a). The contraction of the tissue in response to electrical stimulation was greatly diminished and the release of ATP abolished in the presence of 300 nM TTX (Figure 2b), indicating that these responses were the result of propagated action potentials in nerves and not due to direct electrical depolarization of either the nerve terminals or muscle by the stimulation parameters employed. In 4 experiments, TTX significantly reduced (paired t test on responses in the presence and absence of TTX, $P < 0.05$) the stimulation-induced contractions to 14.2 ± 3.8% (mean ± s.e.mean) and the release of ATP to 8.6 ± 2.0% of their respective controls. The inhibitory effect of TTX on the evoked release of ATP was reversed following washing of the tissue for 55 min (Figure 3c).

Incubation of guinea-pig vas deferens in the presence of the α_1-adrenoceptor antagonist, prazosin (300 nM), greatly diminished the second phase of contraction elicited by nerve stimulation, but did not reduce either the initial twitch response or the release of ATP (Figure 3a). Although this observation and the results with the α_1-adrenoceptor agonist, phenylephrine, indicate that the release of ATP was not due either to muscle contraction or to the postsynaptic action of noradrenaline at α_1-adrenoceptors on the muscle, it still seemed possible that release might arise

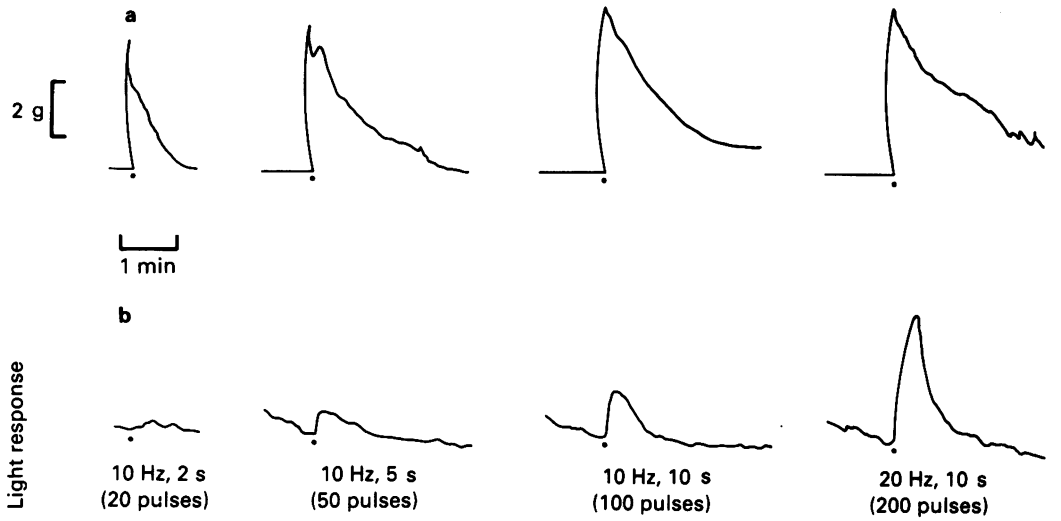

Figure 1 Stimulus-response relationship for contraction and release of ATP from guinea-pig vas deferens. In the example shown, the preparation was incubated in the presence of luciferin-luciferase for 3–5 min and then stimulated with 1 ms pulses at 150 V and the frequencies and durations indicated in the figure. 4-Aminopyridine (300 μM) was present throughout. (a) Shows the force of contraction of the muscle and (b) the release of ATP, indicated by the production of light from the ATP-luciferin-luciferase reaction.

postsynaptically from the muscle as a consequence of the e.j.ps and/or the rapid depolarization produced in the muscle during stimulation of the sympathetic nerves. Previous studies have shown that both the e.j.ps and the rapid twitch responses of guinea-pig vas deferens to either electrical field stimulation or added ATP are diminished following exposure to α,β-methylene ATP (Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984a). These authors assumed that α,β-methylene ATP specifically desensitized the P_2-purinoceptors on the muscle, but this nucleotide has also been shown to inhibit the depolarizations elicited by large concentrations of noradrenaline in the rat basilar artery (Byrne & Large, 1986) and its precise mechanism of action remains to be clarified. In any event, preexposure of the guinea-pig vas deferens to α,β-methylene ATP (100 μM) for two 15 min periods substantially reduced the initial twitch response to electrical stimulation (Figure 3b). On the other hand, pretreatment with α,β-methylene ATP did not diminish the stimulation-induced release of ATP (Figure 3b). In 3 experiments, pretreatment with α,β-methylene ATP and prazosin reduced the maximal contraction to nerve stimulation by over 80% (significantly different from control responses, paired t test, $P < 0.05$), while the release of ATP was reduced by less than 16% which was not statistically different from control (paired t test). From these results, it seems unlikely that ATP was released postsynaptically as a consequence of neurotransmitter-evoked membrane depolarizations in the muscle.

Discussion

The depolarization- and neurotransmitter-induced release of endogenous ATP from sympathetic nerve varicosities isolated from the myenteric plexus of guinea-pig ileum has been demonstrated previously (Al-Humayyd & White, 1985). However, the present study provides the first direct evidence that endogenous ATP is released from sympathetic varicosities as the result of propagated action potentials in the nerves during electrical stimulation. Westfall *et al.* (1978) have previously demonstrated the release of ^{3}H-purines, primarily detected as [^{3}H]-adenosine, following stimulation of guinea-pig vas deferens preloaded with [^{3}H]-adenosine. However, it was not certain whether these ^{3}H-purines reflected released [^{3}H]-ATP or some other nucleotide or nucleoside and also whether release occurred from the nerves or from the smooth muscle. In the present study, stimulation of the non-desheathed vas deferens with a suction electrode released ATP by a TTX-sensitive mechanism, indicating that release was mediated by the generation of propagated action potentials in the sympathetic nerves. Previously, we showed that transmural electrical stimulation of superfused, desheathed guinea-pig vas deferens resulted in a release of endogenous

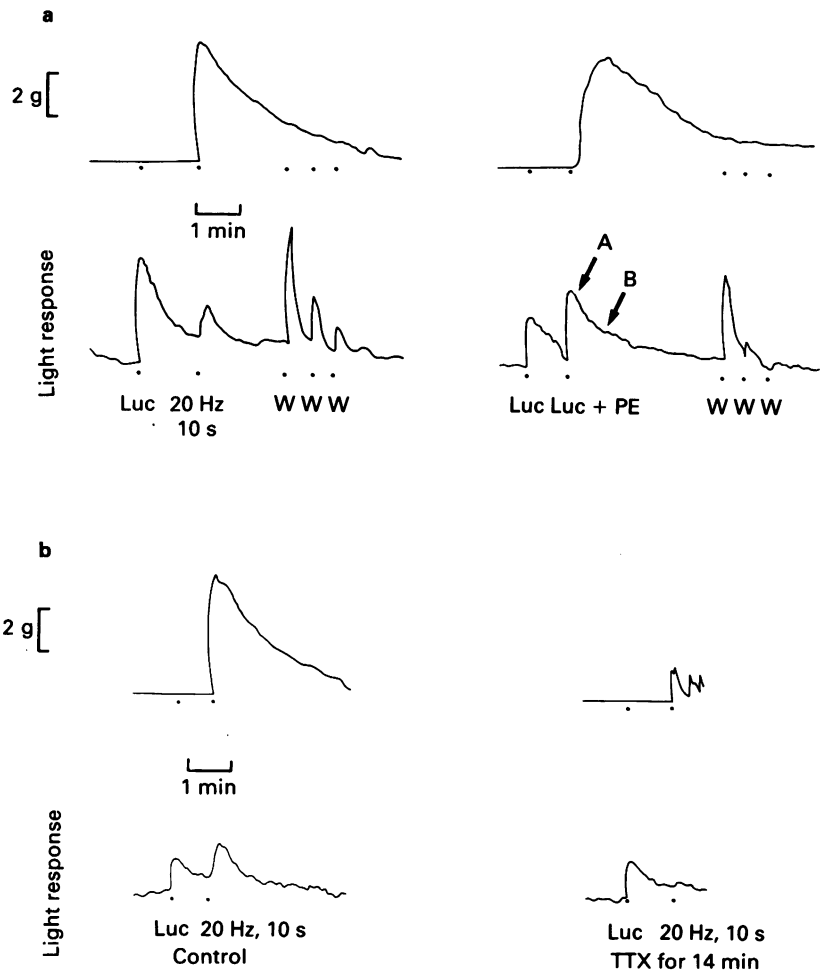

Figure 2 Effect of electrical stimulation, contraction with phenylephrine, and tetrodotoxin (TTX) on the release of ATP from guinea-pig vas deferens. The tissue was prepared and release of ATP detected as described in Figure 1. 4-Aminopyridine was present throughout. (a) The upper tracings show the force of contraction of the tissue and the lower tracings the release of ATP. In the left panel, medium containing luciferin-luciferase was introduced at the point indicated (Luc) and the tissue stimulated with 1 ms pulses at 20 Hz and 150 V for 10 s. 'W' indicates artefacts due to exchanging of medium. In the right panel, medium containing luciferin-luciferase was introduced at the point indicated (Luc), followed 1 min later by luciferin-luciferase medium containing 100 μM phenylephrine (Luc + PE). The tissue began to contract 12 s after the addition of phenylephrine. Note that introduction of medium containing luciferin-luciferase itself produced a light signal. However, at the time of onset of contraction produced by phenylephrine, indicated by (A), and at the time of maximum contraction, indicated by (B), no evoked release of ATP was evident, indicating that contraction of the muscle *per se* is unlikely to account for the release of ATP observed following electrical stimulation of the tissue. (b) The left panel shows the contraction of the muscle (upper tracing) and the release of ATP (lower tracing) evoked by stimulation with 1 ms pulses at 20 Hz and 150 V for 10 s. The right panel shows that incubation of the preparation with TTX (300 nM) for 14 min greatly reduced the stimulation-evoked contraction of the muscle and effectively abolished the release of ATP.

ATP which was not TTX-sensitive, indicating that release was not the result of propagated action potentials in the nerves (White *et al.*, 1981). During transmural electrical stimulation of superfused preparations, which would have relatively small effective bath volumes, the current density could be considerable so that it is possible that both the nerves and the muscle were directly depolarized by the stimulation

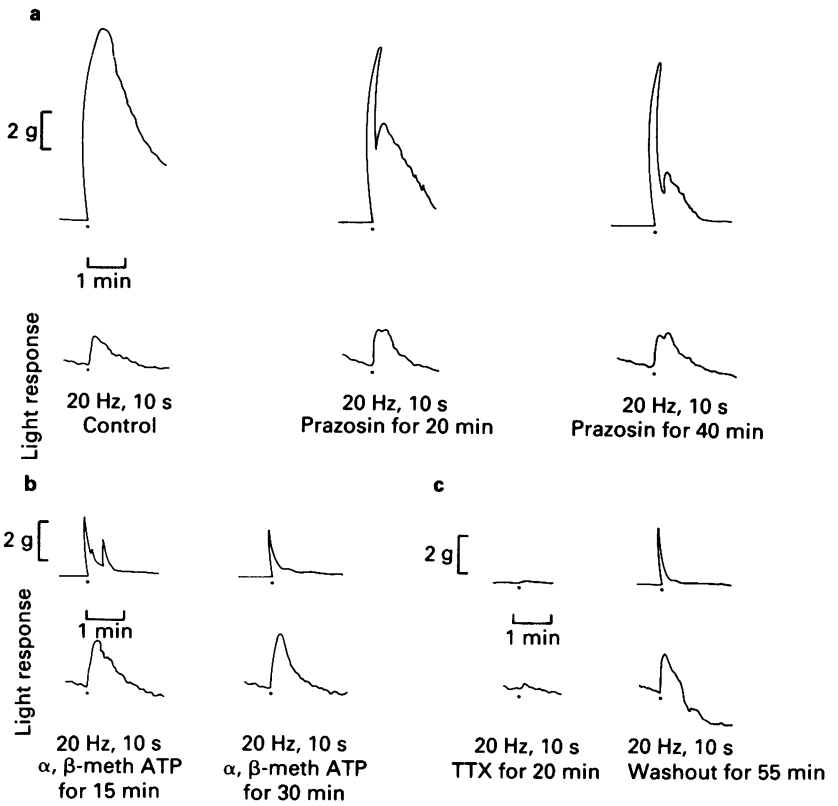

Figure 3 Effect of prazosin, α,β-methylene ATP (α,β-meth ATP) and tetrodotoxin (TTX) on the evoked release of ATP from vas deferens. The tissue was prepared and the release of ATP detected as described in Figure 1. 4-Aminopyridine was present throughout. (a) The left panel shows the contraction of the muscle (upper tracing) and the release of ATP (lower tracing) evoked by stimulation with 1 ms pulses at 20 Hz and 150 V for 10 s. After exposure to the α_1-antagonist prazosin (300 nM) for 20 min, the second, slow phase of contraction was diminished but the release of ATP was unnaffected (middle panel). The second phase of contraction was further reduced after exposure to prazosin for 40 min but release of ATP was still not reduced (right panel), indicating that release was not due to the activation of α_1-adrenoceptors on the muscle. (b) With prazosin present throughout, the preparation was then pre-incubated with α,β-methylene ATP (100 μM) for 15 min, allowed to recover for 8 min with 3 washes and the contraction and release of ATP in response to electrical stimulation re-examined (left panel). The initial, rapid phase of contraction to stimulation was greatly diminished by this treatment and this was further reduced following an additional preincubation with α,β-methylene ATP for 15 min (right panel). In neither case was the release of ATP diminished, suggesting that release of ATP occurred presynaptically from the sympathetic varicosities and not from the muscle. (c) Incubation of the preparation with TTX (1 μM) for 20 min virtually abolished the evoked contraction and release of ATP (left panel). Following washout for 55 min, the rapid twitch response and the release of ATP recovered (right panel). In (c) prazosin was present throughout.

parameters employed.

Stimulation-evoked release of ATP did not appear to arise postsynaptically from the muscle. For instance, release was not due to contraction of the muscle *per se* since contraction by phenylephrine did not release ATP. Moreover, release was not due to the generation of e.j.ps or rapid depolarizations in the muscle during stimulation of the sympathetic nerves since release was not blocked by either the α_1-antagonist, prazosin, or by pre-exposure to α,β-methylene ATP, both of which greatly diminished the postsynaptic responses of the tissue to electrical stimulation. Consequently, it seems most likely that the release of ATP occurred from the sympathetic nerve varicosities innervating the muscle. We cannot say whether ATP was released from sympathetic vesicles or from some other site within the varicosities, nor do we know if the amounts of ATP released were sufficient to produce

the postsynaptic responses observed in the vas deferens. The amounts of ATP detected in the bath were small, but it is likely that only a very small fraction of the released ATP was actually detected due to rapid extracellular degradation and the likelihood that the sheath impairs the diffusion of ATP into the bathing medium, where it can react with the luciferin-luciferase.

Previous studies have shown that the initial depolarizations and rapid twitch responses of guinea-pig vas deferens to sympathetic nerve stimulation are blocked by the ATP analogue, α,β-methylene ATP (Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984b) and by the irreversible P_2-purinoceptor antagonist, arylazido aminopropionyl ATP ($ANAPP_3$) (Fedan *et al.*, 1981; Sneddon *et al.*, 1982), and also that exogenously administered ATP mimics electrical stimulation (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984a; Stjarne & Astrand, 1984). Our evidence of the neuronal release of endogenous ATP provides additional support for the hypothesis that ATP functions as a cotransmitter with noradrenaline in the sympathetic nerves innervating the guinea-pig vas deferens.

Very similar responses to sympathetic nerve stimulation have also been described in blood vessels (Hirst & Neild, 1980; Hirst *et al.*, 1982; Ishikawa, 1985; Kugelgen & Starke, 1985; Muramatsu, 1986; Vidal *et al.*, 1986). Stimulation of sympathetic nerves in certain arterial preparations produces e.j.ps and rapid contractions which are not blocked by α-antagonists; moreover, application of low concentrations of exogenous noradrenaline constricts the vessels by a mechanism which is blocked by α-antagonists but is not associated with changes in smooth muscle membrane potentials. In order to account for these observations, it has been proposed that there are two types of arterial adrenoceptors: (1) extrajunctional α-adrenoceptors at which circulating noradrenaline produces a contraction which is not associated with a change in smooth muscle membrane potential but is blocked by α-antagonists; (2) junctional γ-adrenoceptors at which neuronal noradrenaline produces a contraction by generating e.j.ps which summate to produce an action potential in the smooth muscle (Hirst & Neild, 1980; Hirst *et al.*, 1981). These γ-responses require very large concentrations of noradrenaline and are not blocked by α-antagonists. Recently, several groups have proposed that the junctional responses to sympathetic nerve stimulation in arteries may be due to ATP rather than noradrenaline (Sneddon & Burnstock, 1984b; Ishikawa, 1985; Kugelgen & Starke, 1985; Muramatsu, 1986; Vidal *et al.*, 1986). Their conclusions were based on the ability of ATP to mimic the response to sympathetic nerve stimulation and the blockade by α,β-methylene ATP of responses to both exogenous ATP and nerve stimulation. Moreover, Cheung & Fujioka (1986) have recently reported that the excitatory junction potential in the guinea-pig saphenous artery is inhibited by the irreversible P_2-purinoceptor antagonist, $ANAPP_3$. The specificity of action of α,β-methylene ATP at P_2-purinoceptors has recently been questioned (Byrne & Large, 1986) and release of endogenous ATP has not yet been demonstrated in arteries. However, it is tempting to speculate that release of ATP may be an ubiquitous characteristic of all sympathetic nerves and that ATP may function as a cotransmitter with noradrenaline in a variety of sympathetically innervated tissues.

T.D.W. was a MRC of Canada Visting Scientist at the Baker Medical Research Institute. Research was supported by the MRC of Canada and an Institute Grant from the NH&MRC of Australia to the Baker Institute. We thank Dr J.A. Angus for providing research facilities and his helpful suggestions.

References

AL-HUMAYYD, M. & WHITE, T.D. (1985). Adrenergic and possible nonadrenergic sources of adenosine 5'-triphosphate release from nerve varicosities isolated from ileal myenteric plexus. *J. Pharmac. exp. Ther.*, **233**, 796–800.

AMBACHE, N. & ZAR, M.A. (1971). Evidence against adrenergic motor transmission in the guinea-pig vas deferens. *J. Physiol.*, **216**, 359–389.

BYRNE, N.G. & LARGE, W.A. (1986). The effect of α,β-methylene ATP on the depolarization evoked by noradrenaline (γ-adrenoceptor response) and ATP in the immature rat basilar artery. *Br. J. Pharmac.*, **88**, 6–8.

CHEUNG, D.W. & FUJIOKA, M. (1986). Inhibition of the excitatory junction potential in the guinea-pig saphenous artery by $ANAPP_3$. *Br. J. Pharmac.*, **89**, 3–5.

FEDAN, J.S., HOGABOOM, G.K., O'DONNELL, J.P., COLBY, J. & WESTFALL, D.P. (1981). Contribution by purines to the neurogenic response of the vas deferens of the guinea-pig. *Eur. J. Pharmac.*, **69**, 41–53.

HIRST, G.D.S. & NEILD, T.O. (1980). Evidence for two populations of excitatory receptors for noradrenaline on arteriolar smooth muscle. *Nature*, **283**, 767–768.

HIRST, G.D.S., NEILD, T.O. & SILVERBERG, G.D. (1982). Noradrenaline receptors on the rat basilar artery. *J. Physiol.*, **328**, 351–360.

ISHIKAWA, S. (1985). Actions of ATP and α,β-methylene ATP on neuromuscular transmission and smooth muscle membrane of the rabbit and guinea-pig mesenteric arteries. *Br. J. Pharmac.*, **86**, 777–787.

KUGELGEN, I.v. & STARKE, K. (1985). Noradrenaline and adenosine triphosphate as co-transmitters of neurogenic vasoconstriction in rabbit mesenteric artery. *J. Physiol.*, **367**, 435–455.

MELDRUM, L.A. & BURNSTOCK, G. (1983). Evidence that ATP acts as a co-transmitter with noradrenaline in sympathetic nerves supplying the guinea-pig vas deferens. *Eur. J. Pharmac.*, **92,** 161–163.

MURAMATSU, I. (1986). Evidence for sympathetic, purinergic transmission in the mesenteric artery of the dog. *Br. J. Pharmac.*, **87,** 478–480.

SNEDDON, P. & BURNSTOCK, G. (1984a). Inhibition of excitatory junction potentials in guinea-pig vas deferens by α,β-methylene-ATP: further evidence for ATP and noradrenaline as cotransmitters. *Eur. J. Pharmac.*, **100,** 85–90.

SNEDDON, P. & BURNSTOCK, G. (1984b). ATP as a co-transmitter in rat tail artery. *Eur. J. Pharmac.*, **106,** 149–152.

SNEDDON, P., WESTFALL, D.P. & FEDAN, J.S. (1982). Cotransmitters in the motor nerves of the guinea pig vas deferens: electrophysiological evidence. *Science*, **218,** 693–695.

STJARNE, L. & ASTRAND, P. (1984). Discrete events measure single quanta of adenosine 5'-triphosphate secreted from sympathetic nerves of guinea-pig and mouse vas deferens. *Neuroscience*, **13,** 21–28.

SWEDIN, G. (1971). Studies on neurotransmission mechanisms in the rat and guinea-pig vas deferens. *Acta physiol. scand.*, **Suppl. 369,** 1–34.

THESLEFF, S. (1980). Aminopyridines and synaptic transmission. *Neuroscience*, **5,** 1413–1419.

VIDAL, M., HICKS, P.E. & LANGER, S.Z. (1986). Differential effects of α,β-methylene ATP on responses to nerve stimulation in SHR and WKY tail arteries. *Naunyn-Scmiedebergs Arch. Pharmac.*, **332,** 384–390.

WESTFALL, D.P., STITZEL, R.E. & ROWE, J.N. (1978). The postjunctional effects and neural release of purine compounds in the guinea-pig vas deferens. *Eur. J. Pharmac.*, **50,** 27–38.

WHITE, T., POTTER, P., MOODY, C. & BURNSTOCK, G. (1981). Tetrodotoxin-resistant release of ATP from guinea-pig taenia coli and vas deferens during field stimulation in the presence of luciferin-luciferase. *Can. J. Physiol. Pharmac.*, **59,** 1094–1100.

(Received February 18, 1987.
Revised May 1, 1987.
Accepted June 7, 1987.)