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PURINERGIC INNERVATION OF THE GUINEA-PIG URINARY BLADDER

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1 A number of criteria for considering adenosine 5'-triphosphate (ATP) as a neurotransmitter in the guinea-pig urinary bladder have been examined. In addition, the effect of tachyphylaxis to ATP on the response to non-adrenergic, non-cholinergic nerve stimulation has been re-examined.

2 Quinacrine fluorescence histochemistry revealed a population of nerve fibres, ganglion cells, and nerve bundles in the bladder which were not seen in either the iris or vas deferens, where adrenergic and cholinergic nerves predominate. The distribution and morphology of the quinacrine-positive nerves in the bladder were different from those observed with catecholamine fluorescence and cholinesterase histochemistry, and were unaffected by chemical sympathectomy.

3 Release of ATP from the bladder during stimulation of intramural excitatory nerves, in the presence of atropine and guanethidine increased to 3–12 times prestimulation levels. Tetrodotoxin abolished both the contractile response and the increase in ATP release resulting from intramural nerve stimulation. There was no increase in ATP release during contraction resulting from direct muscle stimulation following nerve paralysis with tetrodotoxin.

4 Sympathectomy with 6-hydroxydopamine did not affect release of ATP in response to intramural nerve stimulation.

5 Release of ATP was dependent on the concentration of calcium ion in the medium.

6 Contractions in response to non-adrenergic, non-cholinergic intramural nerve stimulation were closely mimicked by ATP, but not by acetylcholine or histamine.

7 Adenosine and dipyridamole reduced the contractions to both ATP and non-cholinergic nerve stimulation.

8 2-2'-Pyridylisatogen was not a specific blocker of either ATP or intramural nerve stimulation in the guinea-pig bladder. 2-Substituted imidazolines initiated spontaneous activity making it impossible to assess any blocking action that they may have had.

9 Prostaglandins (E_1, E_2 and $F_{2\alpha}$) gave weak, slow contractions and an increase in spontaneous activity. Both the response to ATP and non-adrenergic, non-cholinergic nerve stimulation were greatly potentiated in the presence of prostaglandins.

10 In the presence of indomethacin the response to non-adrenergic, non-cholinergic nerve stimulation was virtually abolished following desensitization to ATP.

Introduction

The failure of atropine to block the excitatory response of the mammalian urinary bladder to stimulation of sacral parasympathetic nerves has been known for many years (Langley & Anderson, 1895; Henderson, 1923; Henderson & Roepke, 1934; 1935; Ambache, 1955; Ursillo & Clark, 1956; Ursillo, 1961). There have been a number of explanations for this phenomenon. One is that the subjunctional muscarinic receptors present in the bladder are atropine-resistant (Dale & Gaddum, 1930; Elmér, 1975; Carpenter, 1977). Another is that concentrations of atropine capable of blocking exogenous acetylcholine (ACh) are insufficient to compete with high local concentrations of ACh released during cholinergic nerve stimulation (Huković, Rand & Vanov, 1965; Chesher & Thorpe, 1965). Finally the presence of a separate non-cholinergic, non-adrenergic excitatory nerve supply to the bladder has been postulated (Henderson & Roepke, 1934; Chesher & James, 1966; Ambache & Zar, 1970; Dumsday, 1971; Burnstock, Dumsday

& Smythe, 1972), and it has been suggested that adenosine 5'-triphosphate (ATP) is the transmitter released from these nerves (Burnstock *et al.*, 1972), hence termed 'purinergic' (Burnstock, 1972). However, while recognizing that ATP was the only compound of a wide variety tested to mimic closely the nerve-mediated response, Ambache & Zar (1970) did not consider it likely to be the transmitter on the grounds that the nerve-mediated response was not reduced after development of tachyphylaxis to ATP and was often even enhanced.

In the present study, various criteria for the establishment of ATP as a neurotransmitter in the guinea-pig bladder have been examined. These include: (1) storage of ATP, employing the fluorescence histochemical method for the localization of quinacrine (Olson, Ålund & Norberg, 1976) which is known to bind ATP (Irvin & Irvin, 1954); (2) release of ATP during stimulation of intramural nerves; (3) comparison of the time course of the response to nerve stimulation with that to ATP, ACh and histamine; (4) the actions of various drugs on the responses to nerve stimulation and exogenously applied ATP, in particular adenosine; dipyridamole, which potentiates purinergic nerve responses in the intestine (Satchell, Lynch, Bourke & Burnstock, 1972); 2-substituted imidazolines and 2-2'-pyridylisatogen, which are regarded as antagonists of ATP in the intestine (Satchell, Burnstock & Dann, 1973; Spedding, Sweetman & Weetman, 1975).

Finally, we have re-examined the tachyphylaxis induced by high concentrations of ATP, since the previously mentioned experiment of Ambache & Zar (1970) seemed to constitute strong evidence against the proposed role of ATP. We show that tachyphylaxis to ATP is a complicated process involving prostaglandin synthesis. In the presence of an inhibitor of prostaglandin synthesis, tachyphylaxis to ATP is accompanied by profound depression of the neurally evoked response, in a manner consistent with the 'purinergic hypothesis'.

Methods

Pharmacological preparations

Male guinea-pigs (1 day old or adult 300–500 g) were stunned by a blow to the back of the head and exsanguinated. Mucosal-free strips of the detrusor of the bladder (approx. 20 mm × 30 mm) were prepared by the method of Ambache & Zar (1970). Preparations were either superfused with modified Krebs solution (Bülbring, 1953) at 37°C by means of a Watson–Marlow peristaltic pump, or were incubated in Krebs (37°C) in a 50 ml organ bath. All Krebs solutions were bubbled with a 95% O_2, 5% CO_2 gas mixture. Tissues were allowed to equilibrate for 40 to 60 minutes. Isometric tension was recorded on a Grass Model 7D polygraph and a Grass FT10 force-displacement transducer. An initial load of between 0.5 and 1.0 g tension was placed on each preparation. Electrical field stimulation was achieved by means of a pair of platinum ring electrodes approximately 3 mm apart surrounding the tissue strip. Unless otherwise stated, parameters of stimulation were selected which produced responses entirely mediated by nerves (i.e. the response could be blocked with tetrodotoxin). Drugs used were: acetylcholine chloride, adenosine, adenosine 5'-triphosphate disodium and magnesium salts, histamine dihydrochloride, 6-hydroxydopamine, indomethacin, quinacrine dihydrochloride, tetrodotoxin, atropine sulphate (Sigma); dipyridamole (Boehringer Ingelheim); antazoline hydrochloride, guanethidine sulphate, phentolamine mesylate, tolazoline hydrochloride (Ciba); prostaglandins E_1, E_2 and $F_{2\alpha}$ (Upjohn); 2-2'-pyridylisatogen tosylate (gift from M. Hooper). Prostaglandins and indomethacin were dissolved in 70% alcohol as stock solutions (3 mM) and diluted with Krebs solution to the final required concentration. Other drugs were prepared daily as aqueous solutions.

Iontophoretic application of ATP

Thin strips (2 × 4 mm) of detrusor, free of both mucosa and serosa, were stretched and pinned out in a small organ bath containing Krebs solution gassed with 95% O_2, 5% CO_2 and maintained at 37°C. Muscular activity was recorded photoelectrically as described by Purves, Hill, Chamley, Mark, Fry & Burnstock (1974). Magnesium-ATP was applied locally with inward currents to individual smooth muscle bundles by standard iontophoretic techniques.

ATP assay

Fractions of the superfusate were assayed with the firefly luciferin–luciferase reaction which is specific for ATP (McElroy & Seliger, 1963; Strehler, 1968), and the scintillations measured with a Du Pont Luminescence biometer. The reagents were supplied by Du Pont. Sensitivity of the method is 10^{-13} g (0.1 pg) of ATP in samples of 0.01 ml.

Histochemistry

Mucosa-free detrusor preparations were prepared as described above. Iris and vas deferens were dissected free from adult guinea-pigs. All tissues were placed in ice-cold Krebs solution.

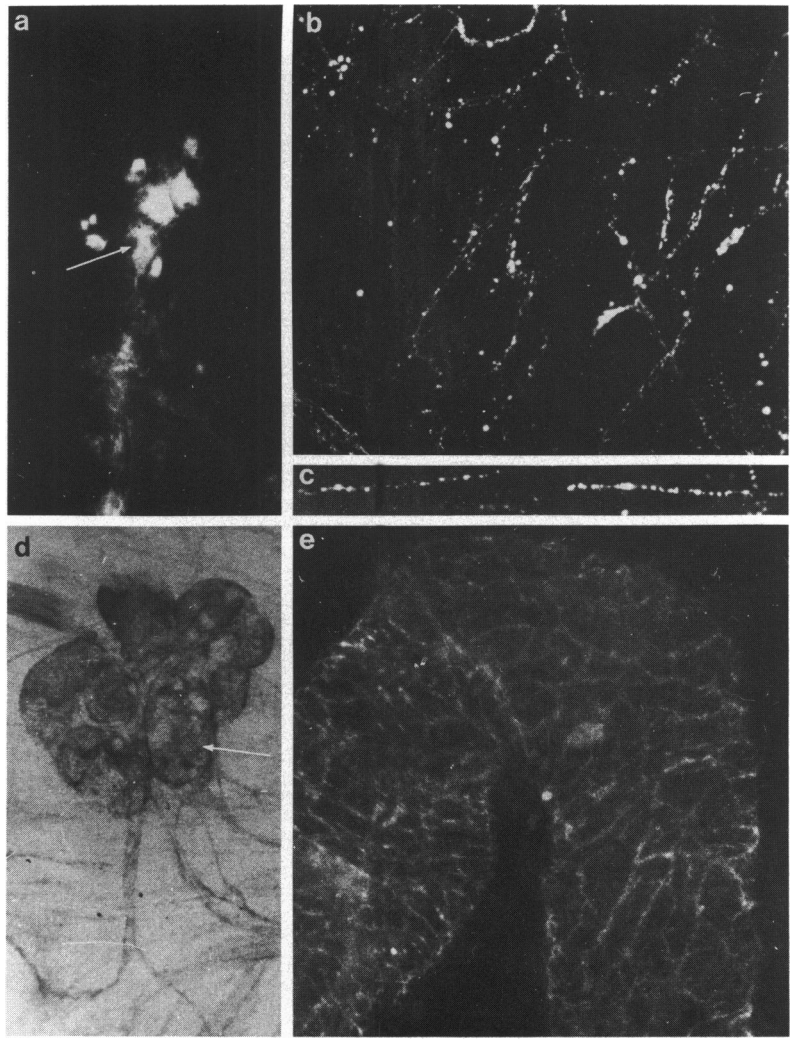

Figure 1 Whole mount stretch preparation of the body of the guinea-pig urinary bladder after removal of the mucosa. (a) Fluorescence histochemical localization of quinacrine: note clusters of fluorescent ganglion cells with distinct non-fluorescent nuclei (arrow) and bundles of nerve fibres with less intense fluorescence. ×200. (b) Fluorescence histochemical localization of quinacrine: a plexus of fine nerve fibres can be seen on the outer surface of the smooth muscle layer. ×200. (c) Fluorescence histochemical localization of quinacrine: note the varicose appearance of fine nerve fibres. ×200. (d) Localization of acetylcholinesterase: ganglion cells (arrow), as well as associated fine nerve fibres can be seen. ×230. (e) Fluorescence histochemical localization of monoamines: note adrenergic nerve fibres form a perivascular plexus about the large looped blood vessel. Very few adrenergic fibres were seen within the smooth muscle of the bladder. ×230.

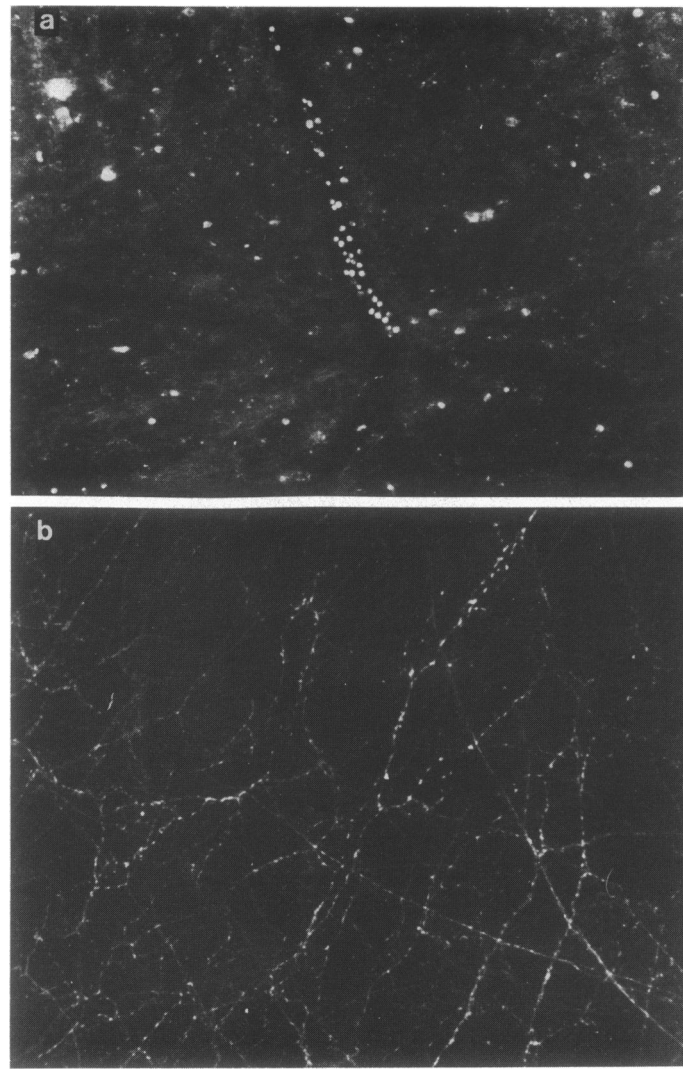

Figure 2 Whole mount stretch preparations of guinea-pig iris. (a) Fluorescence histochemical localization of quinacrine. Nuclei of lymphocytes can be seen within a blood vessel but no fluorescent nerve fibres are present. ×200. (b) Fluorescence histochemical localization of monoamines. Note the dense plexus of fine, varicose nerve fibres. ×200.

Quinacrine fluorescence. Tissues were incubated for 1 h at 37°C in modified Krebs solution (Bülbring, 1953) containing 5×10^{-7} M quinacrine dihydrochloride and bubbled with a gas mixture of 95% O_2, 5% CO_2 (Olson *et al.*, 1976). The tissues were then rinsed thoroughly with quinacrine-free ice-cold Krebs solution. The detrusor and iris were blotted gently with filter paper to remove excess moisture and then stretched on clean glass microslides. Vasa deferentia were frozen in 2-methyl butane cooled in liquid nitrogen and cryostat sections of between 8 to 10 μm were made. The specimens were mounted in liquid paraffin and viewed through a Zeiss photomicroscope fitted with Epifluorescence condensor III RS. This was also suitable for Falck-Hillarp catecholamine fluorescence histochemistry, as the excitation and fluorescence characteristics of the quinacrine molecule are very similar to those of the fluorphores formed by catecholamine and paraformaldehyde (Udenfriend, 1962). Selected areas of the tissues were photographed on Ilford HP4 film.

Monoamine fluorescence histochemistry. Stretch preparations of the bladder and iris were dried over phosphorus pentoxide for 1 h and then treated with gaseous paraformaldehyde (relative humidity 80%) at 80°C for 3 h (Falck, Hillarp, Thieme & Torp, 1962). The vas deferens was first frozen and then freeze dried in a Haake freeze dryer at −30°C and 2×10^{-2} mmHg for 3 days before paraformaldehyde treatment. It was then vacuum embedded in paraffin wax and 10 μm sections prepared. The sections were mounted in liquid paraffin for fluorescence photomicroscopy according to the method described above.

Cholinesterase histochemistry. Whole mounts of the iris, detrusor and fresh cryostat sections of the vas deferens were fixed in formolcalcium for 20 min and stained for cholinesterase as described by Karnovsky & Roots (1964). Selected areas were photographed using Zeiss photomicroscopy on Ilford FP4 film.

Chemical sympathectomy

In some experiments, guinea-pigs were injected intraperitoneally with 6-hydroxydopamine (6-OHDA, 250 mg/kg) 24 h before they were killed. 6-OHDA was dissolved in sterile physiological (0.9% w/v NaCl solution) saline containing 0.2% ascorbic acid.

Results

Storage of ATP

In view of the possibility that quinacrine fluorescence can be used to localize purinergic nerves (see Olson *et al.*, 1976), histochemical studies of whole mount stretch preparations of guinea-pig bladder were carried out with this method and compared with localization of catecholamines and acetylcholinesterase.

Quinacrine fluorescence. Structures resembling nerve fibres exhibited green fluorescence in the detrusor muscle of the urinary bladder of one day old guinea-pigs (Figure 1); atropine fails to block nerve-mediated responses as in the adult. Many of these quinacrine-positive fibres emerged in thick bundles from ganglia which contained quinacrine-positive cell bodies (Figure 1a). A sparse, loose network of quinacrine-stained fibres was also present in the muscle (Figure 1b). The nerve fibres were fine and irregularly beaded (Figure 1c). The background fluorescence of muscle bundles was very low. Details of the nervous elements and ganglia were less clear when the urinary bladder was taken from an adult animal. This was due to the difficulty of preparing sufficiently thin whole mounts of the thicker muscle.

Since there is no evidence that the iris and vas deferens are supplied by purinergic nerves (Burnstock & Costa, 1975), these tissues were used as controls. Cryostat sections of the vas deferens and whole mounts of iris taken from adult animals did not show quinacrine-positive fibres (Figure 2a). There was a low background fluorescence around the choroid, ciliary body and sphincter, a slightly higher background fluorescence around blood vessels, and the nuclei of lymphocytes were intensely stained. In the vas deferens the background fluorescence was lower than that found in the iris.

Catecholamine fluorescence. In the detrusor muscle, adrenergic nerves were few in number compared with quinacrine-positive fibres. Most adrenergic nerve terminals were associated with blood vessels (Figure 1e) although some were found in the muscle. There were no fluorescent ganglion cells associated with bundles of nerve fibres following paraformaldehyde treatment comparable to those seen after quinacrine incubation. Twenty-four hours after injections of 6-OHDA (250 mg/kg) all catecholamine fluorescence was gone, but quinacrine-fluorescent nerves remained. The iris (Figure 2b) and the freeze dried sections of the vas deferens revealed a dense, varicose adrenergic nerve plexus when treated with paraformaldehyde vapour.

Cholinesterase histochemistry. In the detrusor muscle, fine varicose fibres, ganglia and nerve bundles were present (Figure 1d). However, the nerve plexus was more dense and positively-stained neurones within ganglia were more abundant than quinacrine-positive neurones. Furthermore, cholinesterase-positive ganglion cells showed a great variety of shapes whereas quinacrine-positive ganglion cells were always spherical. In addition, cholinesterase-positive

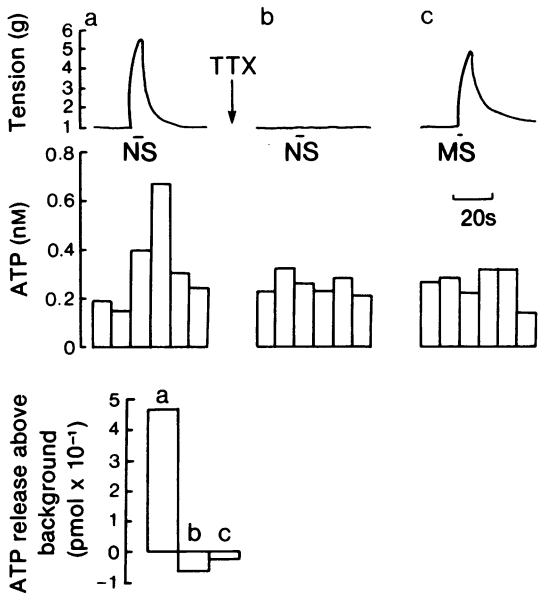

Figure 3 Release of ATP from the superfused guinea-pig bladder strip. Upper trace: mechanical recording of changes in tension (g). Lower trace: concentration of ATP (nM) in consecutive 20 s fractions of the superfusate. (a) Release of ATP during contraction in response to intramural nerve stimulation (NS: 20 Hz, 0.2 ms pulse duration, supramaximal voltage for 10 s). (b) Lack of release of ATP following block of the nerve-mediated response by the addition of tetrodotoxin (TTX, 3.1 μM) to the superfusate 15 min before stimulation. (c) Lack of release of ATP during contraction in response to direct smooth muscle stimulation (MS: 20 Hz, 5.0 ms pulse duration, supramaximal voltage for 2 seconds). Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout. The temperature of the superfusate was maintained between 22°C and 23°C. The bottom figure presents a summary block diagram of the relative ATP release in (a), (b) and (c).

fibres were found in both the iris and vas deferens. Quinacrine-positive nerves were never observed in either of these tissues.

Release of ATP during intramural nerve stimulation

Parameters of electrical field stimulation were selected which produced responses that could be completely blocked by tetrodotoxin, i.e. that were produced via intramural nerves. Conditions for measuring release of ATP from the superfused guinea-pig bladder strip during intramural nerve stimulation in the presence of atropine (1.4 μM) and guanethidine (3.4 μM), were adjusted to give consistent results. If the temperature was kept between 22 and 23°C, and the rate of superfusion between 3 and 4 ml/min, background release of ATP was consistently between 0.12 and 0.19 nM in the superfusate. Under these conditions intramural nerve stimulation (0.2 ms duration pulses delivered at a frequency of 5–20 Hz and at supramaximal voltage for 10 s), caused a 3–12 times increase above background in the release of ATP (10 experiments; see Figure 3a and inset). Increasing the temperature to 37°C gave more variable results; release of ATP during intramural nerve stimulation was between 2 and 20 times that of the background in 4 experiments.

The question of whether the source of ATP released during intramural nerve stimulation is from nerves or secondary, from smooth muscle as a result of changes in tension was examined in 10 preparations. Nerve conduction was completely abolished with tetrodotoxin (TTX, 3.1 μM) which also blocked the accompanying increase in ATP release (Figure 3b). The muscle was then stimulated directly by increasing the duration of the pulses from 0.2 to 5.0 milliseconds. The resulting contraction was similar in both magnitude and time course to that elicited by intramural nerve stimulation, but was not accompanied by an increase in ATP release (Figure 3c). TTX caused a small increase in background release of ATP, but this would not have masked a further increase in ATP release during direct muscle stimulation. Bladder strips from animals sympathectomized with 6-OHDA showed the usual increase in ATP release to intramural nerve stimulation.

The release of transmitters from adrenergic and cholinergic nerves is known to be sensitive to changes in the external calcium ion (Ca^{2+}) concentration (see Triggle & Triggle, 1976) and Holman & Weinrich (1975) have shown that the amplitude of the inhibitory junction potentials in smooth muscle cells of the intestine during stimulation of purinergic nerves is dependent on Ca^{2+} concentration. For this reason the effect of changing the external Ca^{2+} concentration on the release of ATP from the guinea-pig bladder strip was studied and the results are illustrated in Figure 4. Reduction of the Ca^{2+} concentration from 2.5 mM (normal Krebs) to 0.5 mM caused a 30% reduction in the magnitude of the contraction in response to intramural nerve stimulation and a 40% reduction in the ATP released. If the Ca^{2+} concentration was further reduced to 0.25 mM, the contraction became sluggish and greatly reduced in magnitude, and the increase in ATP release above background was reduced by 80 to 90%. Following return to normal Ca^{2+} concentration (2.5 mM), both the mechanical response and the increase in ATP release during intramural nerve stimulation were near control values.

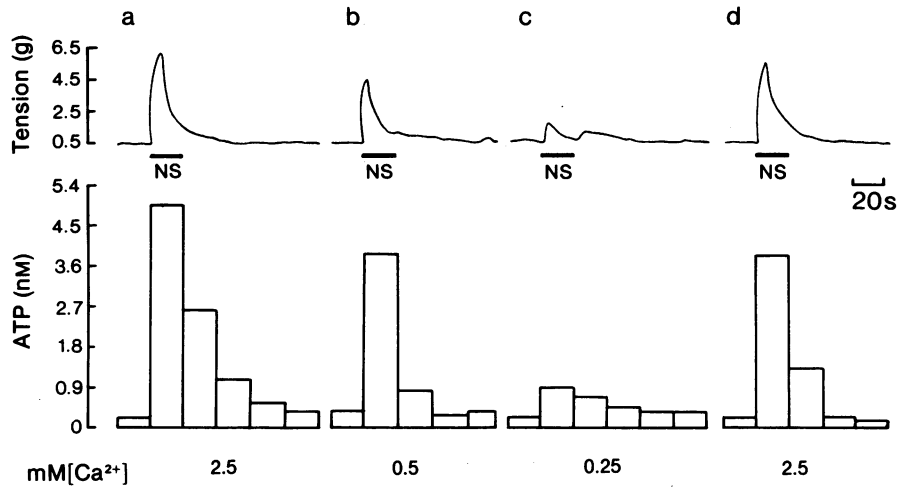

Figure 4 Effect of changing the calcium ion (Ca^{2+}) concentration on the release of ATP from the guinea-pig isolated bladder strip during stimulation of intramural excitatory nerves. Upper trace: mechanical recording of changes in tension (g) during intramural nerve stimulation (NS: 20 Hz, 0.2 ms pulse duration, supramaximal voltage for 20 seconds). Lower trace: concentration of ATP in consecutive 20 s fractions of the superfusate. The Ca^{2+} concentration in the superfusate varied as follows: (a) 2.5 mM (normal Krebs); (b) 0.5 mM; (c) 0.25 mM; (d) 2.5 mM. The successive contractions were separated by 60 min intervals as indicated by the breaks in the mechanical trace. Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout. The temperature of the superfusate was between 22°C and 23°C.

Comparison of the time course of the contractions produced by ATP, acetylcholine, histamine and nerve stimulation

The time course of the contractile response to intramural nerve stimulation in the presence of atropine and guanethidine was compared with that to exogenously applied ATP, ACh (atropine not present) and histamine. In all experiments the response to ATP was found to mimic closely the response to intramural nerve stimulation. These responses consisted predominantly of a rapid phasic contraction which was not maintained despite continuous stimulation (Figure 5a,b). The response to ACh or histamine, however, consisted of a sustained tonic contraction upon which were superimposed regular phasic contractions. Little or no decline in amplitude occurred during maintained stimulation (Figure 5d). Also, the time to reach maximum contraction was greater than that for either ATP or nerve stimulation, and the latency for the responses to both ACh and histamine was between 1 and 3 s compared with about 0.5 s for both ATP and nerve stimulation. As ATP was the only agonist tested which closely mimicked the response to stimulation of non-cholinergic nerves, a more detailed comparison between these two responses was made (Figure 6). Both the rate of contraction and the rate of fade during stimulation were almost identical for contractions of similar magnitude (Table 1). Furthermore, iontophoretic application of ATP to thin strips of bladder (2 × 4 mm) caused rapid dose-dependent contractions of small groups of cells, after a latency of about 1 s (Figure 7).

Effects of adenosine, dipyridamole, 2-2'-pyridylisatogen and 2-substituted imidazolines on ATP and nerve-mediated contractions

Adenosine caused a small decrease in both the tone and spontaneous activity in the bladder. The magnitude of contraction elicited by intramural nerve stimulation (2–5 Hz, 0.2 ms pulse duration at supramaintained stimulation (Figure 5c,d). Also, the time to presence of adenosine. The concentration of adenosine used (between 10 and 100 μM) was chosen in each experiment to give an approximately 20% reduction in this response. In the same concentration range, adenosine also caused a 34 ± 16% (mean ± s.d. from 9 experiments) reduction in the amplitude of contraction elicited by exogenous ATP. The concentration of ATP was such that the contraction was approximately 75% of the contraction due to intramural nerve stimulation.

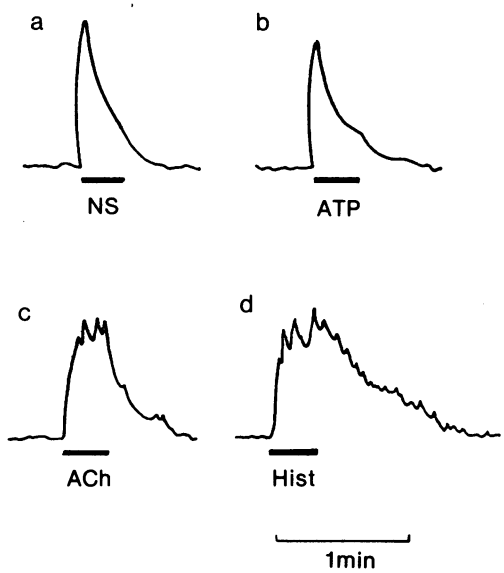

Figure 5 Contractile responses of the guinea-pig bladder strip to: (a) intramural nerve stimulation (NS: 2 Hz, 0.2 ms pulse duration and supramaximal voltage for 20 s); (b) ATP (8.5 μM); (c) acetylcholine (ACh, 2.3 μM) and (d) histamine (Hist, 6.8 μM). All the traces are on the same time scale and sensitivity. The concentration of each agonist was chosen to give a response of similar magnitude to that to intramural nerve stimulation. Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout.

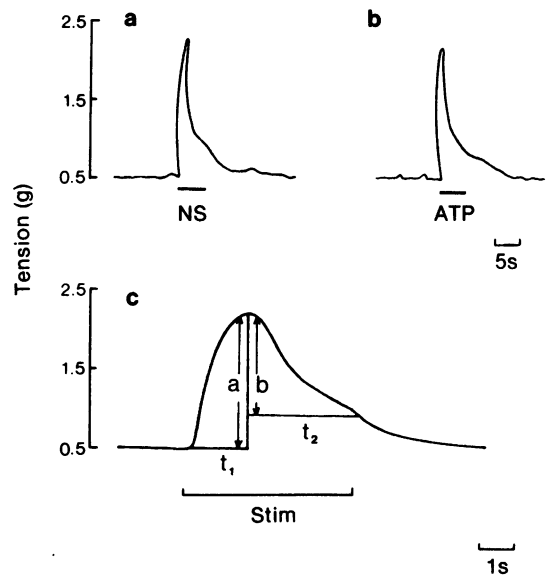

Figure 6 A comparison of the contractile responses of the guinea-pig bladder strip to (a) intramural nerve stimulation (NS: 5 Hz, 0.2 ms pulse duration and supramaximal voltage) and (b) exogenous ATP (8.5 μM). (c) Response to intramural nerve stimulation on a faster time scale, to indicate the basis of the measurements taken: a/t_1 gives the rate of contraction to maximal (g/s) and b/t_2 the rate of 'fade' during stimulation to 25% maximal contraction (g/s) (see Table 1 for values of a/t_1 and b/t_2). Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout.

Table 1 Time course of contraction to intramural nerve stimulation in the guinea-pig bladder compared with that to ATP

Contraction parameters	*Nerve stimulation**	*ATP†*
Magnitude (g)	1.4 ± 0.1‡	1.2 ± 0.4
Rate of increase in tension (a/t_1)¶ g/s	1.3 ± 0.2	1.2 ± 0.3
Rate of fade (b/t_2) g/s	0.3 ± 0.1	0.3 ± 0.1
No. of observations	16	6

* Electrical stimulation of intramural nerves with 0.2 ms duration pulses delivered at 5 Hz and supramaximal voltage for 5 seconds. Atropine (1.4 μM) and guanethidine (3.4 μM) present.
† Concentration of ATP (8.5 μM) chosen to give contractions of similar magnitude to those obtained with intramural nerve stimulation. Atropine (1.4 μM) and guanethidine (3.4 μM) present.
‡ Values given are means with ± s.d.
¶ See Figure 6 for details of both a/t_1 and b/t_2.

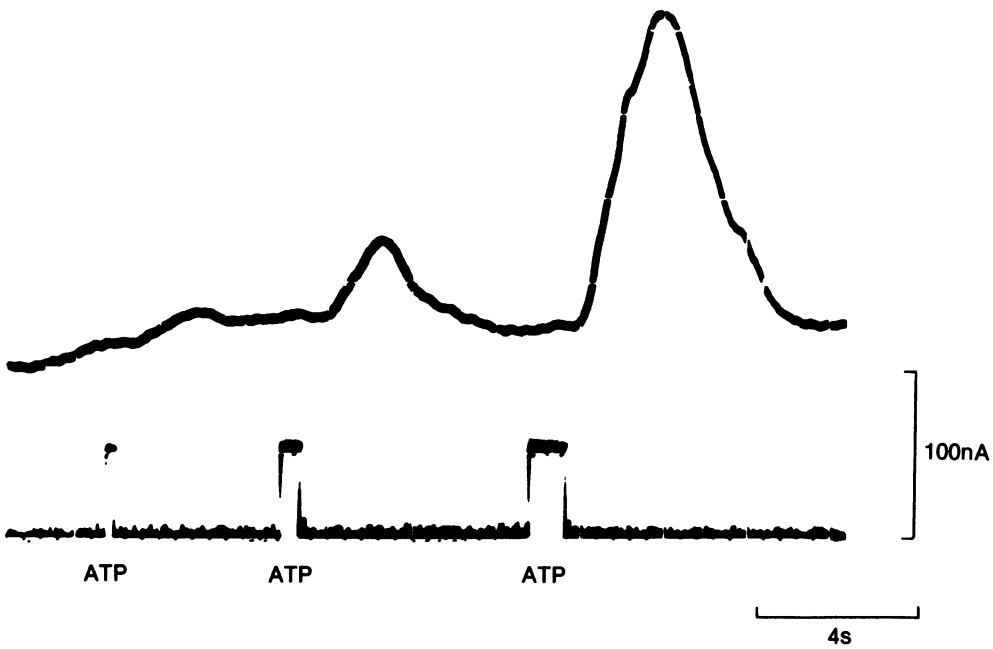

Figure 7 Localized rapid contractions of thin strips of bladder smooth muscle in response to close iontophoretic application of magnesium-ATP. Upper trace: photoelectric record of muscle movement (Purves, Hill, Chamley, Mark, Fry & Burnstock, 1974). Lower trace: iontophoretic current (nA).

Dipyridamole (1.0 μM) caused 31 ± 17% reduction in the magnitude of the response to ATP. The effect of dipyridamole on the response to intramural nerve stimulation depended on the conditions of stimulation. When experiments were carried out in which successive 5 s periods of stimulation were separated by 30 s intervals over a period of 60 to 90 min, a reduction in amplitude of contractions of 40 to 50% was observed with each successive period of stimulation (Figure 8). However, when the interval between successive periods of stimulation was 5 min, reduction of nerve-mediated contractions in the presence of dipyridamole was only about 5% of that of controls. Dipyridamole (1.0 μM) potentiated the effect of adenosine on both nerve- and ATP-mediated contractions.

2-2'-Pyridylisatogen (PIT, 25–50 μM) caused an initial weak, phasic contraction. After 20–40 min exposure to PIT, the 'tone' decreased and spontaneous activity disappeared. Responses to ATP and intramural nerve stimulation were substantially reduced or abolished. However, contractions elicited by histamine and ACh were also reduced in amplitude or abolished by the same concentration of PIT. Lower concentrations of PIT had little effect on the responses to ATP, histamine, ACh or intramural nerve stimulation.

The effect of 2-substituted imidazolines (phentolamine, tolazoline and antazoline) on the responses to nerve stimulation and ATP could not be evaluated because in concentrations 1–50 μM they caused increases in tone and spontaneous activity which altered the sensitivity of the bladder to both nerve stimulation and ATP.

Tachyphylaxis to ATP

The effect of desensitization of the bladder to ATP on the contractions to intramural nerve stimulation (pulses of 0.2 ms duration, frequency 2 Hz, and supramaximal voltage of 30–40 V for 5 s bursts) was studied. Groups of five contractions, each group separated by an interval of 5 or 6 min, were used (Figure 9a). A small decline in the magnitude of contraction both within and between successive groups was always observed over a 40 to 60 min period. Desensitization to ATP was achieved by applying increasing concentrations of ATP to the preparation between successive groups of contractions, ATP being left in the superfusate after each application (Figure 9b). In half of a total of 10 experiments tachyphylaxis to ATP resulted in a significant increase in both the tone and spontaneous activity and the contractions to intramural

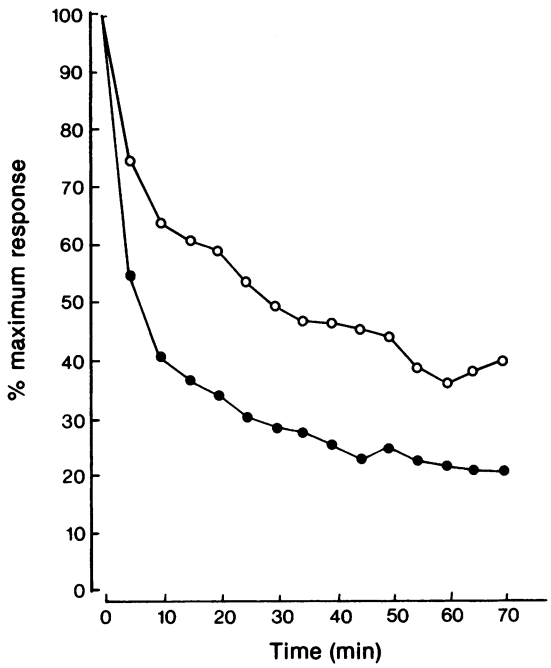

Figure 8 Effect of dipyridamole (1.0 μM) on the contractile response of the guinea-pig isolated bladder to intramural nerve stimulation. Successive contractions over a period of between 60 and 90 min were obtained with 5 s periods of stimulation (5 Hz, 0.2 ms duration and supramaximal voltage) delivered every 30 seconds. (○): Control contractions; (●): similar contractions after treatment with dipyridamole. Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout. In the graph, every 10th contractile response is plotted for the sake of convenience.

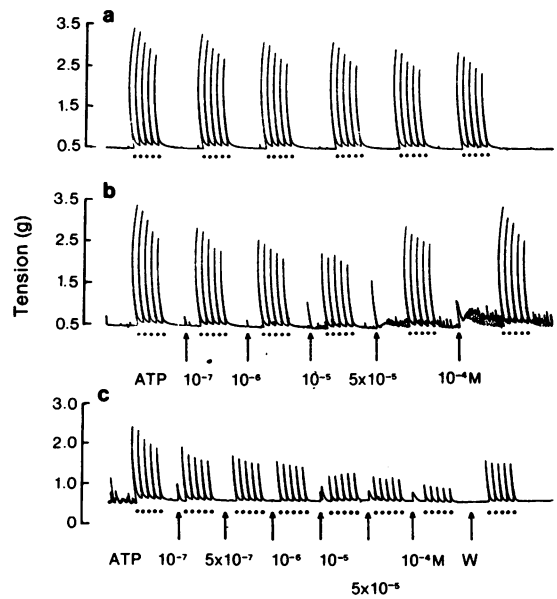

Figure 9 Effect of ATP-desensitization on responses of the guinea-pig bladder strip to intramural nerve stimulation. (a) Control responses showing successive groups of 5 contractions to intramural nerve stimulation (2 Hz, 0.2 ms pulse duration, supramaximal voltage for 5 s) indicated by the black dots. Note the decline in magnitude of contraction both within and between successive groups of contractions. (b) A new preparation and with the same stimulation sequence as in (a), except that between successive groups of contractions cumulative doses of ATP were added in the concentrations indicated (arrows). (c) A new preparation and the same stimulation sequence and ATP applications as in (b). Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout.

nerve stimulation were either unaffected or potentiated (Figure 9b). In the remaining experiments, tachyphylaxis to ATP was not accompanied by an increase in either the tone or spontaneous activity and under these conditions contractions to intramural nerve stimulation were significantly reduced (Figure 9c).

The possibility that the effects of tachyphylaxis to ATP were being obscured by the presence of prostaglandins produced in response to ATP (see Needleman, Minkes & Douglas, 1974) was explored. Prostaglandins E_1, E_2 and $F_{2\alpha}$ (3–30 nM) all gave delayed, sluggish contractions which persisted for up to 10 min after washout. These contractions were often accompanied by an increase in spontaneous activity. In the presence of such low concentrations of prostaglandin E_1, the contractile response to both intramural nerve stimulation and ATP were greatly potentiated (Figure 10). In all preparations pretreated with the prostaglandin synthesis inhibitor indomethacin (2.8 μM) for 60 min, tachyphylaxis to ATP was not accompanied by an increase in either tone or spontaneous activity and there was near-complete blockade of contractions to intramural nerve stimulation (Figure 11). This inhibition of contraction to intramural nerve stimulation was not a result of indomethacin *per se*, since contractions were largely restored after washout of ATP.

Discussion

The results presented here add strong support to the view that non-adrenergic, non-cholinergic excitatory nerves supplying the guinea-pig bladder are puriner-

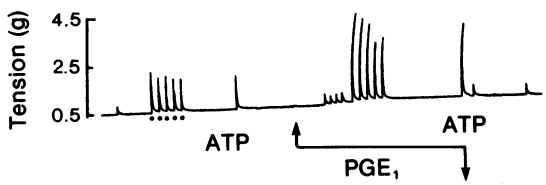

Figure 10 Potentiation of the contractile response of the guinea-pig bladder strip to both intramural nerve stimulation (2 Hz, 0.2 ms pulse duration, supramaximal voltage for 5 s) and exogenous ATP (8.5 μM) by low concentration of prostaglandin E_1 (PGE_1, 28 nM). Note the small but significant increase in both tone and spontaneous activity after addition of PGE_1. Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout.

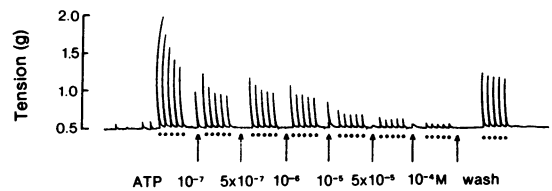

Figure 11 Effect of indomethacin on the response of the guinea-pig bladder strip to intramural nerve stimulation during desensitization to ATP. Responses to intramural nerve stimulation and ATP were obtained as described in Figure 9. Before beginning the stimulation sequence, the preparation was incubated in indomethacin (2.8 μM) for 60 minutes. Note that there is no increase in either tone or spontaneous activity during ATP-desensitization and that the contraction to intramural nerve stimulation is virtually absolished. Atropine (1.4 μM) and guanethidine (3.4 μM) were present throughout.

gic (Burnstock *et al.*, 1972). Evidence for purinergic nervous control of the gastrointestinal tract has been based on the five main criteria generally considered necessary to establish a substance as a neurotransmitter, namely, storage, release and inactivation of the substance, activation of postjunctional receptors, and the action of drugs which modify responses to both nerves and the substance (see Burnstock, 1972; 1975). Thus it is convenient to consider the evidence for purinergic innervation of the bladder under the same headings. In addition, the tachyphylaxis experiment reported by Ambache & Zar (1970) is reinterpreted in view of the results described in this paper.

Storage of ATP

Experiments in which quinacrine fluorescent histochemistry was used (Olson *et al.*, 1976) revealed quinacrine-positive nerve fibres, nerve trunks and ganglia in the detrusor muscle of the guinea-pig urinary bladder. The distribution and morphology of these nervous elements are quite different from those observed with catecholamine fluorescence and are unaffected following degeneration of adrenergic nerves with 6-OHDA. There is some similarity with the plexus observed following cholinesterase histochemistry. However, no nervous elements were seen with quinacrine fluorescence in the iris and vas deferens where catecholamine fluorescence and acetylcholinesterase techniques have revealed both adrenergic and cholinergic nerve fibres. This suggests that quinacrine does not bind to either adrenergic or cholinergic nerve fibres, but to a different population of nerves.

Several separate lines of evidence indicate that quinacrine binds to ATP (Irvin & Irvin, 1954) and to nucleic acids especially DNA (Kurnick & Radcliffe, 1962). The binding involves two consecutive stages and involves two types of complexes; a strong primary binding with high nucleotide phosphate-dye ratios (P/D) and a weaker secondary binding with low P/D ratios (Peacocke, 1973). Although there is ATP present in adrenergic and cholinergic nerve fibres in the iris and vas deferens, the levels may be too low to form a complex with quinacrine. Thus quinacrine may bind only to nerves with high levels of ATP. Intense quinacrine staining has been shown to occur where there are high levels of ATP, e.g. adrenal medulla and platelets. Furthermore, Olsen *et al.* (1976) have found a correlation between the distribution of quinacrine-positive fibres and purinergic nerves in the gut (see Burnstock, 1975). Thus the results of the present studies suggest that quinacrine may be binding to purinergic nerves in the bladder.

This view is supported by recent electronmicroscopic observations of the bladder, in which a population of nerves has been described that contain a predominance of large vesicles (Hoyes, Barber & Martin, 1975), which are similar to 'large opaque vesicles' characteristic of purinergic nerves in the gastrointestinal tract and lung (Robinson, McLean & Burnstock, 1971; Burnstock, 1975). Furthermore, preliminary autoradiographic experiments in our laboratory (Dennison & Burnstock, unpublished observations) show silver grains over nerve profiles containing large opaque vesicles in guinea-pig taenia coli preparations incubated for a brief period (45 s) in low concentrations (0.37 μM) of tritium-labelled adenosine, which appears to be taken up preferentially by purinergic nerves (Su, Bevan & Burnstock, 1971; Rutherford & Burnstock, 1978).

Release of ATP

Release of ATP from intramural nerves supplying the guinea-pig urinary bladder has been demonstrated.

Furthermore, the release of ATP during intramural nerve stimulation was dependent on the concentration of Ca^{2+} in the superfusing medium. A 10-fold reduction of the concentration of Ca^{2+} resulted in reduction of 80–90% of the response to nerve stimulation and a similar decrease in the release of ATP. This supports the conclusion that the ATP is released from nerves since release of neurotransmitters is a Ca^{2+}-dependent process (see Triggle & Triggle, 1976). The findings of Holman & Weinrich (1975) are consistent with this conclusion since they have shown a reduction of more than 80% in the amplitude of inhibitory junction potentials in intestinal smooth muscle cells in response to purinergic nerve stimulation when the external Ca^{2+} was reduced 10-fold.

There are at least four possible sources of neural release of ATP from the bladder; adrenergic, cholinergic, non-adrenergic non-cholinergic and sensory nerves. ATP is known to exist in association with noradrenaline within synaptic vesicles of adrenergic nerves (Burnstock & Costa, 1975). However, adrenergic nerves were eliminated as a source of ATP in this preparation, since chemical sympathectomy with 6-OHDA did not affect either the mechanical response or the accompanying release of ATP on intramural nerve stimulation. Also, the adrenergic neurone blocking drug, guanethidine, was present throughout all release experiments.

Release of ATP together with ACh from motor nerves supplying the rat diaphragm has been reported (Silinsky & Hubbard, 1973). However, there is no evidence for release of ATP in association with ACh from autonomic nerves. On the contrary, Burnstock, Campbell, Satchell & Smythe (1970) demonstrated that in the perfused toad stomach (a preparation in which the 'purinergic'-inhibitory and cholinergic-excitatory components of the vagus can be stimulated independently) release of adenyl compounds could only be detected during 'purinergic' nerve stimulation and not during cholinergic nerve stimulation.

The possibility that ATP is released in response to antidromic stimulation of sensory nerves cannot be excluded (see Holton, 1959), but seems unlikely in view of the lack of evidence for release of ATP from sensory nerve fibres in the gastrointestinal tract (see Burnstock, 1972).

Since release of ATP from intramural nerves supplying the bladder was found to be dependent on temperature and superfusion rate, the localization, availability and activity of ATP-degrading enzymes such as ATPases and non-specific phosphatases may be critical. Lowering the temperature will cause a reduction in activity of these enzymes. However, the amount of ATP released during intramural nerve stimulation will be little affected since it has been shown that the total release of transmitter is little affected by temperature for both motor nerves (Katz & Miledi, 1965) and 'purinergic' nerves in the gut (Jager & Den Hertog, 1974).

Time course of the responses to ATP and intramural nerve stimulation

Previous reports that ATP closely mimics the response to non-cholinergic nerve stimulation in the bladder (Ambache & Zar, 1970; Burnstock *et al.*, 1972) have been confirmed in this study. Furthermore, rapid, dose-dependent contractions to iontophoretically applied ATP suggests that ATP exerts its action directly on the smooth muscle membrane through specific 'purinergic' receptors. The ionic mechanisms underlying the contractile responses of bladder to both ATP and non-cholinergic nerve stimulation are unknown. However, they are both known to produce specific increases in K^+ conductance when producing closely comparable relaxations of intestinal smooth muscle (Tomita & Watanabe, 1973).

Actions of drugs

Few drugs are known which specifically modify the extracellular action of ATP in smooth muscle. In the bladder, the antimalarial drug, quinidine, completely abolishes the contraction to both ATP and intramural nerve stimulation, without blocking the contraction to ACh (Burnstock *et al.*, 1972). The drugs used in this study were chosen as they have been shown to modify purinergic transmission in the mammalian gastrointestinal tract (Satchell *et al.*, 1972; 1973; Spedding *et al.*, 1975). In the guinea-pig taenia coli, dipyridamole, an adenosine uptake inhibitor, potentiates the inhibitory response to both ATP and to purinergic nerve stimulation (Satchell *et al.*, 1972). Both ATP and adenosine cause relaxation in this tissue. If the re-uptake of adenosine is blocked, any adenosine resulting from the degradation of ATP whether released from purinergic nerves or added exogenously, would accumulate extracellularly and potentiate the inhibitory response to both of these stimuli. In the guinea-pig bladder, ATP causes contraction whereas adenosine causes relaxation (Burnstock *et al.*, 1972; this paper). Thus, the responses to both ATP and intramural nerve stimulation are reduced in the presence of dipyridamole. Reduction of the contractions to intramural nerve stimulation by dipyridamole was best observed when successive contractions were separated by 30 s rather than by 5 minutes. This is probably because with 30 s intervals, adenosine derived from neurally released ATP will tend to accumulate within the tissue.

PIT, which has been claimed to be a specific ATP antagonist in the guinea-pig taenia coli (Spedding *et al.*, 1975), was not found to be so in the bladder. The use of 2-substituted imidazolines as antagonists

to ATP in the gastrointestinal tracts (see Satchell *et al.*, 1973) could not be effectively tested in the bladder. This was due to large increases in spontaneous activity during incubation with these drugs, which altered the sensitivity of the bladder to both nerve stimulation and ATP. Therefore, quinidine appears to remain the most effective antagonist to both ATP and purinergic nerve responses of the bladder to date (see Burnstock *et al.*, 1972).

Tachyphylaxis to ATP

A major criticism against ATP being the neurotransmitter of non-cholinergic excitatory nerves supplying the guinea-pig urinary bladder was the finding of Ambache & Zar (1970), that upon desensitization of the bladder to ATP, the contractile response to non-adrenergic, non-cholinergic nerve stimulation was unaffected, or even potentiated. Interpretation of this result (confirmed here) is complicated by the occurrence of a significant increase in both the tone and spontaneous activity of the preparation during the experiment. Since prostaglandins have been demonstrated in this paper to increase tone and spontaneous activity, and since there is evidence that ATP induces prostaglandin synthesis (Needleman *et al.*, 1974; Burnstock, Cocks, Paddle & Staszewska-Barczak, 1975), the experiment was repeated in the presence of the prostaglandin synthesis inhibitor, indomethacin. Under these conditions, there was no increase in tone or spontaneous activity, and during tachyphylaxis to ATP, the response to intramural nerve stimulation was almost abolished. Thus the failure to demonstrate a decrease in the nerve-induced response during tachyphylaxis in the absence of indomethacin seems likely to be due to the presence of prostaglandins and a consequent increase in the sensitivity to intramural nerve stimulation. However, it is difficult to explain why, under these conditions, tachyphylaxis to exogenous ATP is not also prevented.

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