J. Physiol. (1970), **210**, *pp.* 761–783
With 7 text-figures
Printed in Great Britain

NON-CHOLINERGIC TRANSMISSION BY POST-GANGLIONIC MOTOR NEURONES IN THE MAMMALIAN BLADDER

BY N. AMBACHE AND M. ABOO ZAR

From the Medical Research Council, Department of Physiology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2

(*Received 22 April 1970*)

SUMMARY

1. In mucosa-free preparations of the detrusor muscle electrical stimulation with 0·1 msec pulses has been utilized to reveal the non-cholinergic nature of most, if not all, of the post-ganglionic motor neurones in the guinea-pig bladder.

2. The twitches elicited by 0·1 msec pulses were abolished by tetrodotoxin, but were not reduced by dimethyltubocurarine or by hexamethonium. Hexamethonium was nevertheless present in all the experiments in order to restrict acetylcholine action to 'muscarinic' receptors in the muscle fibres.

3. There was little or no diminution in the twitches after prolonged exposure to atropine, 10^{-8}–10^{-5} g/ml., although the twitch-matching dose of acetylcholine was raised 1000–2500 times. Hence, there was no anomalous refractoriness to atropine in these 'muscarinic' receptors.

4. Despite massive atropinization, the recruitment of unoccupied transmitter-receptors by means of extra pulses remained unaffected.

5. Eserine failed to potentiate the atropine-resistant twitches.

6. The twitches were not depressed by morphine.

7. Noradrenaline produced relaxation and twitch reduction. The twitches persisted after $\alpha+\beta$ adrenoceptor blockade with phentolamine + pronethalol and were unaffected by the monoamine oxidase and catecholamine-*O*-methyl transferase inhibitors, tranylcypromine and pyrogallol.

8. Rapid contractions were elicited by 5-HT and by histamine but the twitches remained unaltered after antagonism of 5-HT by methysergide and of histamine by mepyramine.

9. The twitches could be mimicked by injections of ATP, but after desensitization of the preparation to ATP the response to electrical stimulation remained unaltered.

10. Prostaglandins E_2 and $F_{2\alpha}$, only in large doses, produced delayed, sluggish contractions which persisted after the wash; these contractions were quite different from the responses to electrical stimulation, which were immediate, sharp and brief.

11. The non-cholinergic nature of the post-ganglionic motor neurones was confirmed in the bladder of two other species, the cat and the rabbit.

INTRODUCTION

The partial atropine-resistance of the contractions induced in the bladder by stimulation of its parasympathetic nerve supply was first noted by Langley & Anderson (1895), who placed their electrodes on the sacral nerve roots. With the recognition of cholinergic transmission at many parasympathetic synapses the post-ganglionic transmitter in the bladder was believed to be acetylcholine (ACh). But the refractoriness to atropine, noted again by Henderson & Roepke (1934, 1935), was puzzling, and its existence in all species investigated has prevented full acceptance of cholinergic transmission at these endings. Various attempts have been made to explain away this atropine-resistance, in order to retain uniformity with the rest of the parasympathetic nervous system, in which the post-ganglionic transmitter is known to be ACh. The implications of this anomaly have been discussed at length in a review article (Ambache, 1955), which concluded that 'the nature of the parasympathetic post-ganglionic neurones in the bladder remains unsolved' and in which the alternative explanation, of a non-cholinergic transmission for this and some other atropine-resistant nerve effects, as envisaged by Henderson & Roepke (1934), was considered. This problem has now been re-examined and the results presented in this paper provide several converging lines of evidence that the post-ganglionic motor innervation of the guinea-pig bladder is, in fact, predominantly non-cholinergic.

In all previous investigations of the neurohumoral transmission in the bladder large numbers of pulses have been used to elicit each contraction (25, Edge, 1955; 60–150, Ursillo & Clark, 1956; 100, Ursillo, 1961; 40, Chesher & Thorp, 1965, Figs. 4 and 5; 10–200, Huković, Rand & Vanov, 1965; 100–200, Vanov, 1965; 75–450, Edvardsen, 1968). It has been claimed that the non-extinction of bladder contractions by atropine was due to a flooding of receptors in the muscle fibres by an excess of released ACh, which was able to overcome the atropine block (Huković *et al.* 1965; Edvardsen, 1968). We have therefore used single pulses to reduce the amount of transmitter to a minimum, but still find atropine virtually ineffective.

It has also been thought that the 'muscarinic' ACh receptors in the

muscle fibres of the bladder behave anomalously towards atropine or may even be inaccessible to this drug. We have examined this second possibility by following the decline in sensitivity to ACh during prolonged soaking in atropine, 10^{-8}–10^{-5} g/ml.; the observed decrease in ACh sensitivity (1000 2500 ×) shows that the 'muscarinic' ACh receptors are no less susceptible to atropine in the bladder than elsewhere. The experiments were conducted in hexamethonium, because the antagonism of ACh by atropine cannot be evaluated properly unless the 'nicotinic' ACh receptors in the intramural ganglia and nerve endings have been put out of action by means of a ganglion-blocking agent; moreover, the electrically induced responses are then due to a pure excitation of the post-ganglionic neurones, uncomplicated by possible additional ganglionic effects of ACh released as a result of the inevitable co-excitation of preganglionic fibres. However, under these conditions, overspilling preganglionic ACh might still diffuse to the 'muscarinic' receptors in the muscle fibres; this process would be augmented by eserine. Potentiation by eserine in the absence of atropine has been noted by other workers and taken as evidence for cholinergic transmission at the post-ganglionic endings. But atropine would eliminate any such contribution from preganglionic ACh to the 'muscarinic' response, which would then be due entirely to the action of the post-ganglionic transmitter. Our results show that, after the total elimination of the two possible 'preganglionic' contributions by means of hexamethonium plus atropine, the persisting twitch response to single pulses is not potentiated by eserine at all, despite the ready availability of unoccupied transmitter-receptors, shown by the fact that an extra pulse can produce a large increment in the response.

A preliminary account of these results, which were demonstrated to the Physiological Society, has appeared previously (Ambache & Zar, 1970*b*).

METHODS

Male albino guinea-pigs weighing 0·6–1 kg were killed by concussion and bled out. Micturition often occurred on opening the peritoneal cavity but, when necessary, the exposed bladder was emptied by syringe, the needle puncture being placed as low as possible. The bladder was held lightly at its apex, stretched gently, and the investing layer of serosa, connective tissue and fat, with its visible blood vessels and nerves, was lifted with fine forceps and dissected away *in situ* with curved scissors, as close to the convex outer surface of the bladder as possible. The bladder was excised by a low transverse cut above the trigone; residual urine was absorbed on filter paper, and the tissue was washed in a Petri dish with several changes of Locke's solution at room temperature. The bladder was then taken out and collapsed between index finger and thumb into a flat pouch, which was opened by two lateral incisions and unfolded to give a rectangular sheet of tissue some 1·5–2·5 cm long (unstretched) and 0·7–1 cm wide. Blunt separation of the mucosa, visible as a looser superficial pink layer, was started at one end and continued by traction with Hudson's

strabismus forceps, repeatedly grasping the full width of the mucosa; clean removal of the mucosa was usually possible, without fraying or tearing of the underlying muscle. The muscle was flattened with the convex side of the scissors and any loose tags were cut. The step of removing the mucosa was considered essential for improving the oxygen supply to the preparation and for providing better access, on both sides of the thin muscle sheet, for administered drugs. The width of this rectangular sheet of tissue was then reduced to 4–5 mm by trimming and straightening the edges; an estimate of its thickness was obtained on the discarded trimmings, which were slid back and forth between the anvils of a micrometer screw gauge until slowed by friction; this assessment, in a few preparations, indicated a thickness of 0·4–0·8 mm. A silk ligature was sewn, at the top and bottom of the preparation, about 1 mm from each cut end, and knotted as a 2–3 mm loop, to avoid squeezing the ends on suspension. From smaller bladders a 'double length preparation' was tailored, by slitting the rectangular sheet of muscle longitudinally down the middle for all but the last 3 mm at one end, which were left to act as a connecting link between the two halves when they were extended for suspension as a continuous strip.

From 2·3–3·3 kg male cats and rabbits 40 × 3 mm strips were cut over the apex of the bladder, and freed from serosal and mucosal coverings.

The preparations were maintained at 35° C in Krebs–Henseleit solution, gassed in the reservoir and in the organ bath with a 95 % O_2–5 % CO_2 mixture. The composition of this solution and the design of the 2 ml. organ bath with built-in vertical platinum electrodes have been given previously (Ambache & Freeman, 1968). The tap was kept slightly open throughout the experiment to allow a gentle flow of the nutrient fluid through the bath, but was closed during drug contacts. Muscle contractions were recorded with Kavanagh's (1962) lever, at a magnification of × 12. In some early experiments the load was varied until an optimum was found, at which the contraction recorded in response to a constant stimulus became maximal; on the basis of this experience loads of 0·2–1·6 g, depending upon the size of the muscle, were used in subsequent experiments. The loaded preparations lengthened to 3·5–5 cm; some were weighed at the end of the experiment (180–270 mg).

The organ bath electrodes were connected to an electronic stimulator capable of delivering 500 mA (Bell, 1968). We are indebted to Mr P. M. G. Bell, Department of Pharmacology, Oxford, for modifications to this stimulator, which allow, by dial setting, the delivery of an exact number of pulses up to 100 at a preselected frequency up to 100 Hz. The preparation was stimulated at supramaximal voltage (27 V) with 1–5 rectangular pulses delivered at regular intervals usually of 50 or 60 sec, but in a few experiments 30, 33 or 100 sec. After preliminary trials at 0·1–0·5 msec, the pulse width was fixed at 0·1 msec for most of the experiments and the frequency at 10 Hz. Pulse delivery was checked in two ways: acoustically, from the audible click emitted with each pulse by the loudspeaker in the stimulator; and visually, on the numeral indicators of a TC-11 Time Counter (Advance Electronics Ltd., Hainault, Essex).

Drugs. Dosages of the following substances mentioned in the text refer to their respective salts, which were: ACh chloride, adenosine-5′-monophosphate sodium salt (AMP), adenosine-5′-diphosphate sodium salt (ADP), adenosine-5′-triphosphate disodium salt (ATP), S-adenosyl-(−)-methionine chloride, atropine sulphate, bretylium tosylate, dimethyltubocurarine bromide, di-isopropyl fluorophosphonate (DFP), dopamine hydrochloride, eserine sulphate, hexamethonium bromide, histamine dihydrochloride, hyoscine hydrobromide, 5-hydroxytryptamine creatinine sulphate, mepyramine maleate, methysergide bimaleate, morphine sulphate, neostigmine methyl sulphate, (−) noradrenaline bitartrate, pentolinium tartrate, phentolamine mesylate and pronethalol hydrochloride.

Adenosine 3′:5′-cyclic-monophosphoric acid (cyclic AMP), γ-amino-*n*-butyric acid and prostaglandins were used as the free acids; and (−)-Dopa (3-hydroxytyrosine) and tranylcypromine (2-phenylcyclopropylamine), as the free bases.

We are indebted to Dr J. E. Pike, Upjohn Ltd., Kalamazoo, Michigan, for PGE_2 and $PGF_{2\alpha}$; to Dr J. H. Sanner, Searle & Co., Chicago, Illinois, for SC-19220 (1-acetyl-2-(8-chloro-10,11-dihydrodibenz [b, f] [1,4]oxazepine-10-carbonyl) hydrazine); to Dr D. F. Cole, Institute of Ophthalmology, London, for polyphloretin phosphate; and to KABI, Pharmaceuticals, Stockholm, for emepronium (ethyl (3,3-diphenyl-1-methyl-propyl) dimethylammonium) bromide.

RESULTS

Evidence for non-cholinergic post-ganglionic transmission in the guinea-pig bladder

Single pulse stimulation in hexamethonium-treated preparations

The isolated detrusor muscle preparations were excited at 30–60 sec intervals with single rectangular pulses of supramaximal voltage and 0·1–0·3 msec duration. The resulting twitch response was unaffected by ganglion-blocking doses of hexamethonium (10^{-4} g/ml.). Most of the experiments were, nevertheless, conducted in the presence of this ganglion-blocking agent, thus eliminating any possible contribution of 'nicotinic' ACh receptors to the response. The twitch produced by single 0·1 msec pulses was completely abolished (Fig. 1, T) by tetrodotoxin, 2×10^{-7} g/ml. Therefore, with this pulse width, the response is due entirely to the excitation of conducted action potentials in the post-ganglionic neurones. However, when the pulse width was increased to 0·3 msec, it was found that a small response-remnant survived even after tetrodotoxin had completely paralysed the twitches elicited by the 0·1 msec pulses. The slightly longer pulses thus appear to exert an additional effect on the preparation, possibly at the nerve-endings, where these strong currents may cause a leakage of transmitter. For the following experiments the pulse width was therefore fixed at 0·1 msec throughout.

Atropine-resistance. In its rapid onset this bladder contraction is reminiscent of the twitch elicited by single, brief pulses in the guinea-pig ileum (Paton, 1955), which is undoubtedly due to the release of ACh. The bladder twitch reaches its peak within 1 sec; relaxation is slower (Fig. 6*A*) and its duration depends upon the load. With lightly loaded levers, the duration of the twitches in the two preparations is not very different. Records obtained on fast drums in the present experiments showed that the bladder twitch lasted some 10–20 sec, whilst in the experiments of Ambache & Freeman (1968) on the separated longitudinal muscle of the ileum the twitches lasted 3–8 sec. However, whereas in the ileum the post-ganglionic motor neurones are cholinergic and the twitch is completely

abolished by atropine, $0 \cdot 4-1 \times 10^{-8}$ g/ml., or by hyoscine, in the bladder the twitch response persisted, little changed, even after the preparations had been left to soak for several hours in atropine, $10^{-8}-10^{-5}$ g/ml., and its duration was unaltered. The reduction in the height of the response by atropine, due to the non-availability of the 'muscarinic' receptors to neurogenic ACh, whether of pre- or of post-ganglionic origin, was at most 20 % (e.g. *ca.* 17 % in the experiment of Fig. 3), but usually < 10 %. When this partial reduction of the twitch response by atropine happened, it was noticed that all of the reduction occurred with 10^{-8} or 10^{-7} g/ml. and that there was then no further reduction when the atropine concentration was raised 10 or 100 times, to 10^{-6} and 10^{-5} g/ml. In several experiments there was no reduction whatsoever or even a slight increase in twitch height, for instance in the experiment of Fig. 1, which we will now consider in detail.

It has been argued that the 'muscarinic' ACh receptors in the bladder behave anomalously towards atropine. The sensitivity to ACh was therefore checked at frequent intervals before and during exposure to atropine in this and in several other experiments, by determining the dose of ACh required to produce a contraction matching the twitch response. Thus, before atropine was introduced in the experiment of Fig. 1, the twitches (identified by the contraction height marked 1 in panel *A*) were matched by the immediate response to ACh, $0 \cdot 1$ μg/ml., shown at *B*; this contraction was not fully maintained and the ACh was washed out after 2 min contact. This twitch-matching dose of ACh will now be referred to as x, and all subsequent doses of administered ACh will be expressed as multiples of x.

After the preparation had been exposed to atropine 10^{-8} g/ml. for 20 min, the dose of ACh required to match the unaltered twitch response

---

Legend to Fig. 1.

Fig. 1. Atropine causes no diminution and eserine no potentiation of the contractions due to post-ganglionic stimulation. Guinea-pig bladder strip in hexamethonium, 10^{-4} g/ml.; load, $0 \cdot 8$ g. The twitches (response level marked 1 in panel *A*) were elicited at 33 sec intervals by single rectangular $0 \cdot 1$ msec pulses of supramaximal voltage. The larger responses, marked 2–5, were elicited by trains of 2, 3, 4 or 5 pulses, respectively ($0 \cdot 1$ msec; 10 Hz). At the white dots, ACh injected and left in the bath for $0 \cdot 5-3$ min; its dosage is expressed as multiples of $x = 0 \cdot 1$ μg/ml., the twitch-matching dose before atropine (panel *B*). Exposure to atropine, consecutively: 50 min in 10^{-8} g/ml. (starting 17 min before panel *C*); 62 min in 10^{-7} g/ml. (starting 30 min before *D*); 59 min in 10^{-6} g/ml. (starting 31 min before *E*); and 63 min in 10^{-5} g/ml. (starting 28 min before *F*). Eserine, $2 \cdot 5 \times 10^{-6}$ g/ml., for 21 min from the first arrow in *F*. At T, tetrodotoxin, 2×10^{-7} g/ml. Details in text.

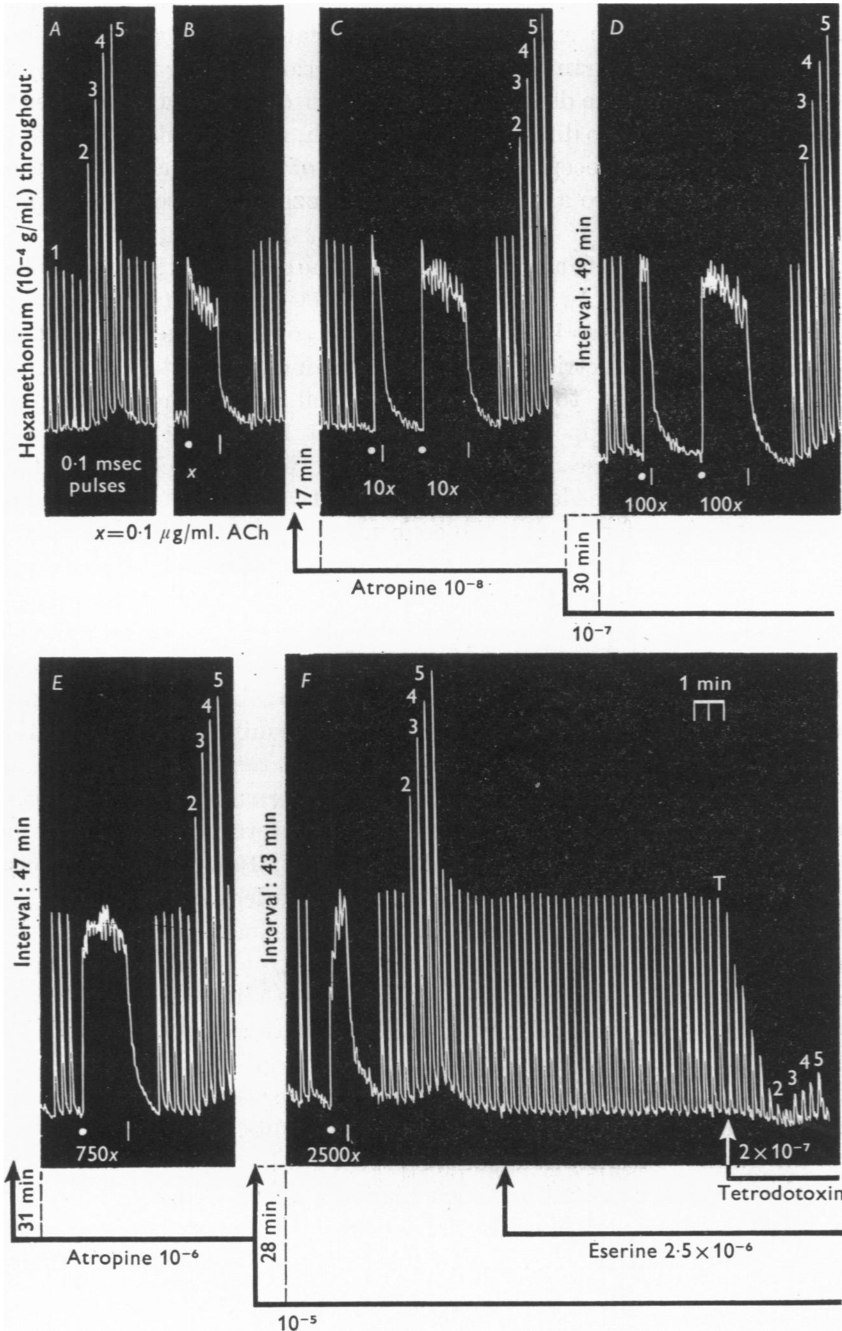

Fig. 1. For legend see opposite page.

was now found to be $10x$. As shown in panel C, this dose of ACh was washed out after 30 sec and repeated 3 min later, when the $10x$ dose of ACh was left in the organ bath for a longer period in order to allow more time for diffusion into the deeper parts of the muscle. But the longer 3 min exposure to ACh made no difference to the match, and it would appear that the 'muscarinic' ACh receptors in the depth of this muscle, altogether some 0·5 mm thick, were as effectively atropinized as those in its superficial layers.

After 50 min exposure to atropine 10^{-8} g/ml. the concentration of atropine was raised to 10^{-7} g/ml. As shown in panel D, 33–37 min later the twitch-matching dose of ACh was $100x$, irrespective of contact time; on the other hand, the response to electrical stimulation remained completely unaltered. After 62 min exposure to atropine, 10^{-7} g/ml., the concentration of atropine was again stepped up ten times, to 10^{-6} g/ml.; 34 min later (panel E) twitch responses were still unaltered, but a dose equivalent to ACh, $750x$, was now needed to match these twitches. After soaking in atropine, 10^{-6} g/ml., for 59 min, the concentration of atropine was raised, finally, to 10^{-5} g/ml. Panel F shows the sluggish response, 31 min later, to ACh, $2500x$, stabilizing to a plateau height that matched the twitch response, which was still unreduced after some 4 hr in atropine.

Other atropine-like drugs. In experiments conducted at first in atropine alone (10^{-7} g/ml.), the subsequent inclusion of hyoscine, 10^{-6} g/ml., in the bath fluid for up to 1·8 hr did not produce any significant change in the responses to single pulses.

It has been claimed that emepronium, a quaternary ammonium base with parasympatholytic properties, is a more powerful relaxant drug in the bladder than atropine (Jönsson & Zederfeldt, 1957). However, in a concentration of $2{\cdot}5 \times 10^{-6}$ g/ml., it also failed to affect the twitches.

These results make it difficult to invoke inadequate blockade of the 'muscarinic' ACh receptors in the bladder by atropine and other anti-muscarinic drugs as a reason for the persistence, with little or no reduction, of the responses to electrical stimulation. The sometimes total insensitivity of the twitch to atropine raises serious doubts as to the cholinergic nature of the post-ganglionic motor neurones in the bladder. The following results with morphine only serve to accentuate these doubts.

Morphine. In concentrations of 10^{-6}–10^{-5} g/ml., morphine failed to reduce the atropine-resistant twitches elicited by single pulses.

Dimethyltubocurarine. This drug in a concentration of 30 μg/ml. produced a slight potentiation of the twitches and an increase in rhythmic activity.

Repetitive stimulation with 2–5 pulses

In the experiment of Fig. 1 a further test was carried out on the preparation, from time to time. As shown in panels *A* and *C–F* (responses identified by numerals 2–5), the effect was also recorded of repetitive stimulation at 10 Hz, with trains of, respectively, 2, 3, 4 or 5 pulses, each of 0·1 msec width. These responses, too, were virtually abolished by tetrodotoxin (panel *F*) but were not reduced by atropine; in fact, as with the twitch, their amplitude increased slightly (cf. panels *C–F* with panel *A*).

Throughout this and other similar experiments the response height was capable of considerable growth as the number of pulses was increased. For instance, means obtained from the contractions marked 2–5 in panels *C–F*, i.e. after atropinization, show an increase in response height (over that of the single-pulse twitch) of, respectively, 50, 78, 94 and 106 %. In this experiment, as the interval between the groups of stimuli was only 33 sec, there was a distinct but slight rise in the base line between the tetanic contractions. Therefore, in four other experiments conducted in hexamethonium, 10^{-4} g/ml., and atropine, 10^{-7} g/ml., in two of which hyoscine, 10^{-6} g/ml., was also present for 0·5 hr before the determinations, a longer interval of 60 or 100 sec was adopted, to ensure complete relaxation between the tetanic contractions. The results are illustrated graphically in Fig. 2. The growth in response amplitude with an increasing number of pulses was even more striking than in the experiment of Fig. 1. In two of these experiments the results were identical whether the interval between the groups of stimuli was fixed at 60 or at 100 sec, showing that with 60 sec intervals the increments had not been due to residual excitability changes in the muscle. Again, it made no difference to the results whether the determinations were carried out soon after atropine was introduced or several hours later. Thus the difference between the three curves in the left hand panel of Fig. 2 is not due to the differences in the duration of exposure to atropine. For these three experiments, the mean percentage increments over the single-pulse twitch height were: for 2 pulses, 77·1 %; for 3 pulses, 168·9 %; for 4 pulses, 235·6 % and for 5 pulses, 279·3 %. In another experiment (no. 4 of Fig. 2) the percentage increments for 2–4 pulses were determined both before (graph *A*) and after (graph *B*) the administration of atropine and hyoscine; with 5 pulses the response was offscale and could not be measured. Without atropine or hyoscine, the percentage increments were: for 2 pulses, 86·1 %; for 3 pulses, 173·8 %; and for 4 pulses, 192·2 %. After 2 hr in atropine, 10^{-7} g/ml., during the last 0·5 hr of which hyoscine, 10^{-6} g/ml., was also present, the percentage increments had become slightly greater, i.e. 115·5, 203·3 and 258·4 %, respectively. This accounts for the slight shift of the fourth curve to the left (*A* to *B*)

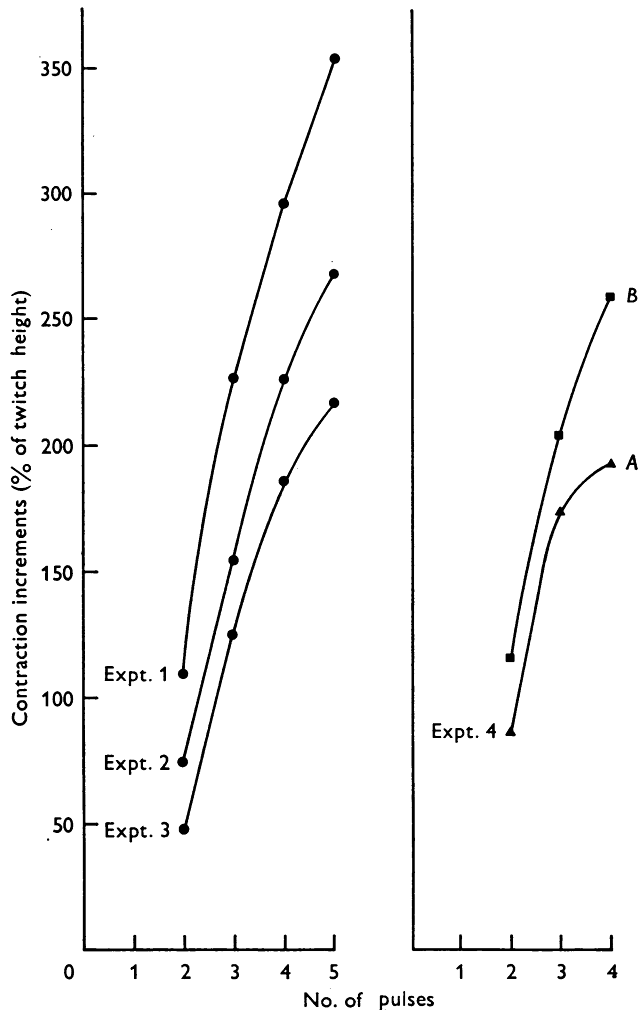

Fig. 2. Percentage increase in response, in excess of twitch height, with increasing number of pulses, delivered at 10 Hz. Results obtained at different loads but constant magnification from four guinea-pig bladder preparations treated with hexamethonium, 10^{-4} g/ml., and atropine, 10^{-7} g/ml. Groups of stimuli were spaced at 100 sec intervals, except in expt. 2 (60 sec). Further details in text. *Left panel*: Expt. 1, after 1·5 hr in atropine. Expt. 2, after 3 hr in atropine. Expt. 3, after 4·8 hr in atropine, with hyoscine, 10^{-6} g/ml., also present during the last 30 min. *Right panel*: Expt. 4; graph *A* was determined before, and *B* after, 2 hr in atropine, with hyoscine, 10^{-6} g/ml., also present during the last 30 min. In this experiment, the response to 5 pulses was offscale and is not shown.

in atropine and hyoscine, which is the opposite of what would be expected if the transmitter were ACh.

It is evident from these results that one or more extra pulses have the capacity of producing a very considerable increase in the response, presumably through a 'recruitment' of unoccupied transmitter-receptors in the muscle by the additional quanta of released transmitter. The availability to the transmitter of these unoccupied receptors remains unimpaired even after prolonged soaking in high concentrations of atropine and hyoscine.

Yet, despite this ready availability, cholinesterase inhibitors, even in massive doses, were incapable of producing any recruitment whatsoever of such unoccupied receptors by the unknown motor transmitter, as shown in Fig. 1 (panel F) and described more fully in the following section.

Absence of potentiation by cholinesterase inhibitors in the presence of atropine

It could be held that the atropine-resistant bladder twitch was due to ACh still able to act upon receptors which, for some unknown reason, remain inaccessible to the blocking action of atropine. If that were so, eserine should protect this ACh from destruction by cholinesterases and so potentiate the twitch, just as it does other known cholinergic nerve effects.

However, in the bladder preparations, once atropine was present, eserine or neostigmine failed to produce any increase in twitch height, even when given in concentrations which, in other tissues, have been found adequate to inhibit completely both types of cholinesterase. For instance, in the experiment of Fig. 1 (panel F) eserine, $2{\cdot}5 \times 10^{-6}$ g/ml., was left in the organ bath for 21 min but did not produce the slightest change in the twitches. In another experiment, which was conducted in a lower concentration of atropine (10^{-6} g/ml.) eserine, 10^{-6} g/ml., again failed to potentiate the responses to electrical stimulation but lowered the twitch-matching dose of ACh from $1000x$ to $200x$, thus demonstrating the presence and operation of cholinesterase(s) in the preparation. And in a third experiment, conducted in atropine, 10^{-7} g/ml., but (exceptionally) without hexamethonium, there was, after 23 min exposure to eserine, $2{\cdot}5 \times 10^{-6}$ g/ml., no increase whatsoever in the twitch height or in the responses to 2–5 pulses, which showed the usual recruitment.

On the other hand, if eserine was administered before the preparations were atropinized, it gave rise to changes attributable to its anticholinesterase action. The difference between the effectiveness of eserine before and after atropine is illustrated by the experiment of Fig. 3, conducted in the presence of hexamethonium. Panel A shows the response to single 0·1 msec pulses and the usual recruitment with 2–5 pulses. As shown in panel B, when eserine, 2·5 μg/ml., was left in the organ bath for 5 min it produced

a gradual rise in the tone of the preparation, associated with an increase in the height of the twitches; and some spontaneous rhythmic activity was now present between the twitches. At the end of the 5 min contact the contracted state of the muscle raised the twitch peaks to the level previously achieved by 3 or 4 pulses in panel *A*. The effect of eserine persisted for a considerable time after it was washed out. It was due to protected ACh, of either pre- or post-ganglionic origin, acting at the 'muscarinic'

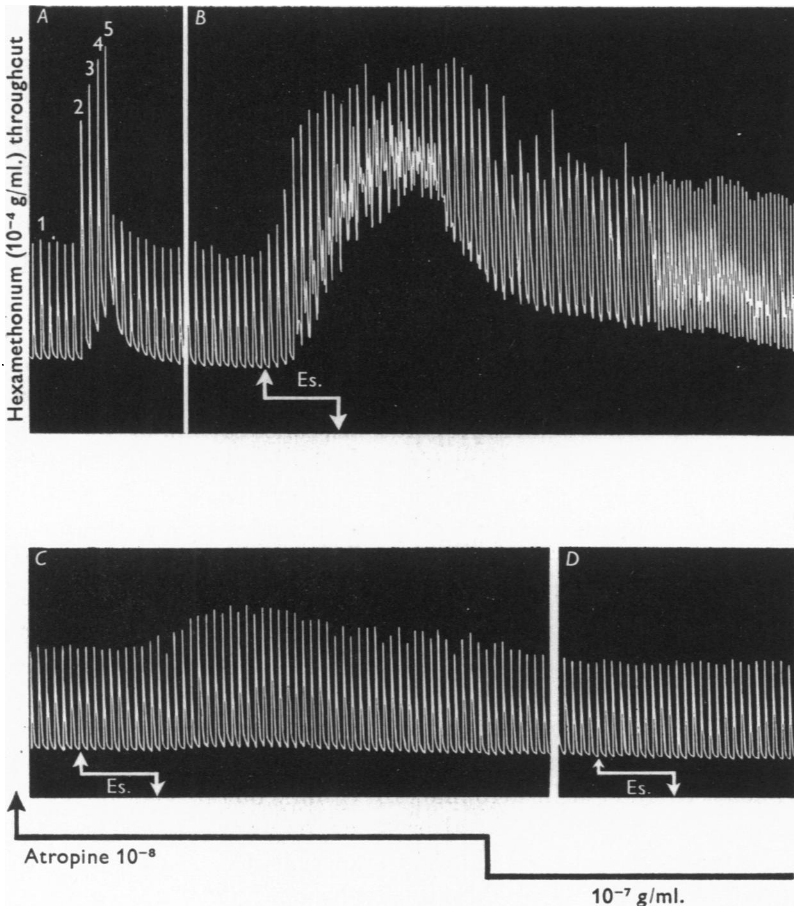

Fig. 3. Effect of eserine before and after atropine. Guinea-pig bladder strip in hexamethonium, 10^{-4} g/ml. Twitches were elicited at 33 sec intervals by single 0·1 msec pulses of supramaximal voltage (response level marked 1 in panel *A*); the larger responses marked 2–5 were elicited by trains of 2–5 pulses (0·1 msec; 10 Hz). In *B*, *C* and *D*, eserine (Es.), 2·5 μg/ml., left in the bath for 5 min between the arrows. Atropine, 10^{-8} g/ml., was introduced 28 min before the eserine in panel *C*, and increased to 10^{-7} g/ml. 20 min before the eserine in panel *D*.

receptors, because it was abolished by atropine. Thus, following 28 min exposure to atropine, 10^{-8} g/ml., the administration of eserine produced no effect during the 5 min contact period, though after the eserine was washed out some potentiation of the twitches still occurred (panel *C*). But 20 min after the concentration of atropine was increased to 10^{-7} g/ml., eserine produced no effect whatsoever, either during the 5 min contact period or afterwards (panel *D*).

The specific inhibitor of acetylcholinesterase, BW 284C51, 1:5-bis-(*p*-allyldimethylammoniumphenyl)-pentan-3-one dibromide, 5×10^{-6} g/ml., failed to potentiate the atropine-resistant twitch responses, as also DFP, a preferential inhibitor of butyrylcholinesterase, in a dose range of 0·02–20 μg/ml. (10^{-7}–10^{-4} M).

Exclusion of some known active substances as the possible post-ganglionic transmitter in the bladder

Catecholamines

Elliott (1905, 1907) described the presence in two species, the ferret and the frog, of motor adrenoceptors in the detrusor muscle of the bladder. However, in the present experiments on guinea-pigs, adrenergic motor transmission to the detrusor muscle could be excluded because noradrenaline, 0·5–1·5 μg/ml., produced a relaxation of the preparations and a reduction in the height of the atropine-resistant twitches; moreover, in the absence of atropine, the contractile effect of ACh was greatly reduced by noradrenaline.

Dopamine and (–)-Dopa (3-hydroxytyrosine) were both inactive in concentrations of 1·5 μg/ml.

Adrenergic transmission was further excluded by the findings that the twitches persisted after $\alpha + \beta$ adrenoceptor blockade with phentolamine (10^{-6} g/ml.) plus pronethalol (10^{-6} g/ml.), and after exposure of the preparation to the adrenergic-neurone blocking agent, bretylium, 2×10^{-6} g/ml. Lastly, the twitch height remained unchanged in the presence of the monoamine oxidase inhibitor, tranylcypromine, 10^{-6} g/ml., and of the catecholamine-*O*-methyl transferase inhibitor, pyrogallol, 10^{-6} g/ml.

5-Hydroxytryptamine

5-HT contracted the atropinized preparations, but its role as a possible post-ganglionic transmitter was excluded by means of the specific 5-HT antagonist, methysergide, as illustrated in Fig. 4. In this experiment a 'double length' preparation from a small bladder was used, lightly loaded; atropine and hexamethonium were present throughout. Before methysergide the twitches, elicited by single 0·1 msec pulses, were matched (panel *A*) by 5-HT, 0·15 μg/ml., a dose which will be called *y*. The pre-

paration was then exposed to methysergide in a concentration of, at first, 10^{-9} g/ml. for 72 min, and subsequently 10^{-8} g/ml. During this exposure the twitch response underwent little or no diminution, although the sensitivity to 5-HT fell greatly, and its twitch-matching dose rose to 50 y after 54 min in the methysergide, 10^{-9} g/ml., (panel *B*) and finally to 400 y after 41 min in 10^{-8} g/ml. (panel *C*).

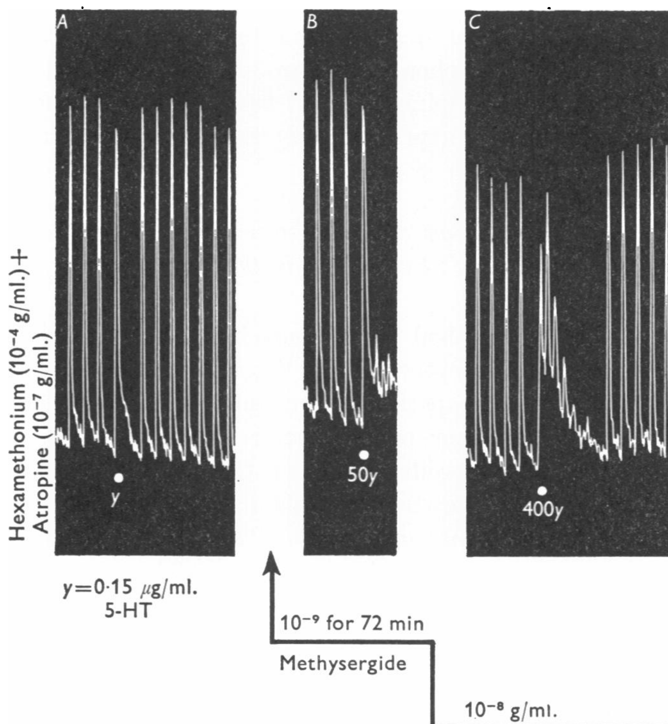

Fig. 4. Persistence of twitch responses after 5-HT blockade. Guinea-pig bladder 'double length' preparation in hexamethonium, 10^{-4} g/ml., and atropine, 10^{-7} g/ml.; load 0·2 g. All twitches were elicited at 1 min intervals by single 0·1 msec pulses of supramaximal voltage. At the white dots, 5-HT administered for 1 min; its dosage is expressed in multiples of y = 0·15 μg/ml., the twitch-matching dose before methysergide (panel *A*). In *B*, the twitches persist and are matched by 50 y, after 54 min in methysergide, 10^{-9} g/ml. Methysergide concentration raised to 10^{-8} g/ml. at the end of 72 min. In *C*, the twitches remain unaltered and are matched by 400 y, after 41 min exposure to the methysergide 10^{-8} g/ml.

Histamine

Histamine produced sharp contractions of the detrusor muscle and in several sensitive preparations the twitch-matching dose was as low as 0·1 μg/ml. However, histaminergic transmission at the post-ganglionic endings

could be excluded because the twitch responses remained unaffected by the histamine antagonist, mepyramine in experiments conducted in the presence of hexamethonium and atropine. One of these is illustrated in Fig. 5. The initial twitch height can be seen in panel *A*, and its match by histamine, 0·1 μg/ml., which will be referred to henceforth as dose *z*. Mepyramine, 10^{-8} g/ml., abolished the response to *z* (panel *B*) after 20 min

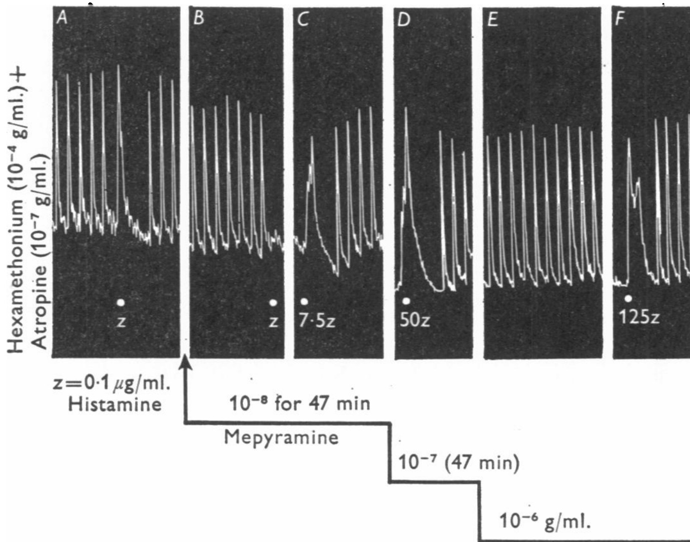

Fig. 5. Persistence of twitch responses after histamine blockade. Guinea-pig bladder, 'double length' preparation in hexamethonium, 10^{-4} g/ml., and atropine, 10^{-7} g/ml.; load 0·2 g. All twitches were elicited at 1 min intervals by single 0·1 msec pulses of supramaximal voltage. At the white dots, histamine administered for 20 sec in *A* and for 1 min elsewhere; its dosage is expressed in multiples of z = 0·1 μg/ml., the twitch-matching dose before mepyramine (panel *A*). In *B*, *z* is ineffective after 20 min in mepyramine, 10^{-8} g/ml. In *C*, twitches matched by 7·5 *z*, 3 min later. In *D*, twitches matched by 50 *z*, after 30 min in mepyramine, 10^{-7} g/ml. In *E*, twitches still unaltered after 10 min in mepyramine, 10^{-6} g/ml. In *F*, twitches matched by 125 *z*, after 25 min in mepyramine, 10^{-6} g/ml.

and, 3 min later, raised the twitch-matching dose of histamine to 7·5 *z* (panel *C*). Following 47 min exposure to 10^{-8} g/ml., the mepyramine concentration was increased to 10^{-7} g/ml., which raised the twitch-matching dose of histamine to 50 *z* after 30 min (panel *D*). At the end of 47 min exposure to 10^{-7} g/ml., the mepyramine concentration was increased to 10^{-6} g/ml. The undiminished twitch responses to electrical stimulation 10–20 min later are shown in panel *E* and the twitch-matching dose, now 125 *z* after 25 min, in panel *F*. Subsequently, after 43 min exposure to 10^{-6} g/ml. the mepyramine concentration was raised still further, to 10^{-5}

g/ml., but this produced a marked rise in tone and the appearance of spontaneous contractions.

In mepyramine-treated plexus-containing preparations of the longitudinal muscle from the guinea-pig ileum histamine 0·1 μg/ml. exerts a strong inhibitory effect upon atropine-resistant 'tetanic spasms' (Ambache & Zar, 1970*a*; Ambache, Verney & Zar, 1970). In the present experiments on bladder preparations an inhibitory effect of this kind, after exposure to mepyramine, could not be detected and twitch height remained unaltered in the presence of histamine, 0·1 μg/ml.

Amino acids

In the dose range $0{\cdot}25–5 \times 10^{-5}$ g/ml. DL-alanine, γ-aminobutyric acid, L-arginine, D-aspartic acid, neutralized DL-glutamic acid, L-histidine, glycine, L-lysine and DL-serine were all inactive.

ATP and other adenosines

Of all the substances tested ATP was the only one which seemed to reproduce closely the effect of electrical stimulation, in that it elicited an immediate sharp contraction whose shape and duration were comparable with those of the twitch response (Fig. 6, recorded on a fast drum). With the other spasmogens already considered, the contractions were maintained with little or no decline for the whole of the contact time; but with ATP the contraction subsided quite rapidly, despite the continued presence of the ATP in the organ bath (Fig. 6). The electrically induced twitch could be matched in height by ATP, 1–5 μg/ml., but the matching dose did not remain constant because of marked tachyphylaxis to successive doses of ATP (Fig. 7). With large doses of ATP the sharp contraction was followed by a rise in the tone of the preparation, which often persisted after the ATP was washed out.

The brief response to ATP appeared to resemble the electrically induced twitch so closely as to suggest that ATP might be triggering some process essential to the twitch. However, the action of ATP was not due to the initiation of nerve impulses in the preparation, because it persisted after the twitch response to electrical stimulation was paralysed by tetrodotoxin, 2×10^{-7} g/ml.

The unknown motor transmitter in the bladder cannot be identical with ATP, because the atropine-resistant twitches elicited by single pulses remained unaltered during the progressive desensitization of the preparation to ATP by tachyphylaxis. This is illustrated in Fig. 7. In this experiment electrically induced twitches were alternated at 1 min intervals with 30 sec contacts to various doses of ATP. Panel *A* shows that the twitch response was matched initially by the first dose of ATP, 1 μg/ml., and that the response to the same dose repeated subsequently became progressively

smaller. In panel *B*, the response to a 3 μg/ml. dose of ATP was now only about one third of the twitch height and the dose had to be raised to 10 μg/ml. to obtain a match; on repetition, the response to this higher dose of ATP underwent marked tachyphylaxis. In panel *C*, ATP had become totally ineffective in its initial dose of 1 μg/ml. but a match was obtained with the second of the two 25 μg/ml. doses and later, in panel *D*, with 100 μg/ml. The preparation was then further desensitized by a continuous

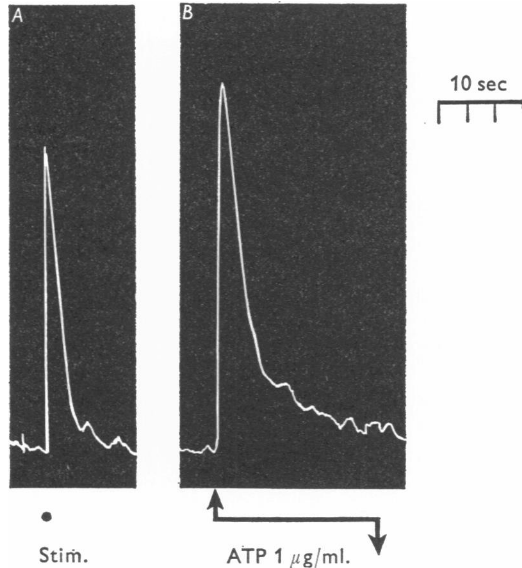

Fig. 6. Imitation of the twitch response by ATP. Guinea-pig bladder, 'double length' preparation; hexamethonium, 10^{-4} g/ml., atropine, 10^{-7} g/ml. and hyoscine, 10^{-6} g/ml. Load 0·2 g; fast drum. In panel *A*, twitch elicited by a single 0·1 msec pulse of supramaximal voltage. In panel *B*, the rapid contraction produced by an injection of ATP, 1 μg/ml.; although the ATP was left in the bath for 1 min, between the arrows, the contraction was not maintained.

5 min exposure to 100 μg/ml., followed for 30 sec by a superimposed equal dose of ATP, making a total of 200 μg/ml. On washing out, it was found that, as a result of this treatment, there was now (panel *E*) a virtual extinction of the response to the third dose of ATP, 100 μg/ml., and no response at all to a 10 μg/ml. dose.

It can be seen in Fig. 7 that, throughout this experiment, there was no diminution whatsoever in the electrically induced twitch responses at any time, either during the tachyphylaxis or after the further desensitization with the massive doses of ATP.

ADP and AMP appeared to be 10 and 200 times less active, respectively,

than ATP. Cyclic AMP, 1–100 μg/ml., and S-adenosyl-methionine, 10 μg/ml., were completely inactive.

Prostaglandins

With prostaglandins it was not possible to imitate the rapid twitch which is evoked by electrical stimulation. The sensitivity of the preparations to the two most active prostaglandins of the *E* and *F* series, PGE_2

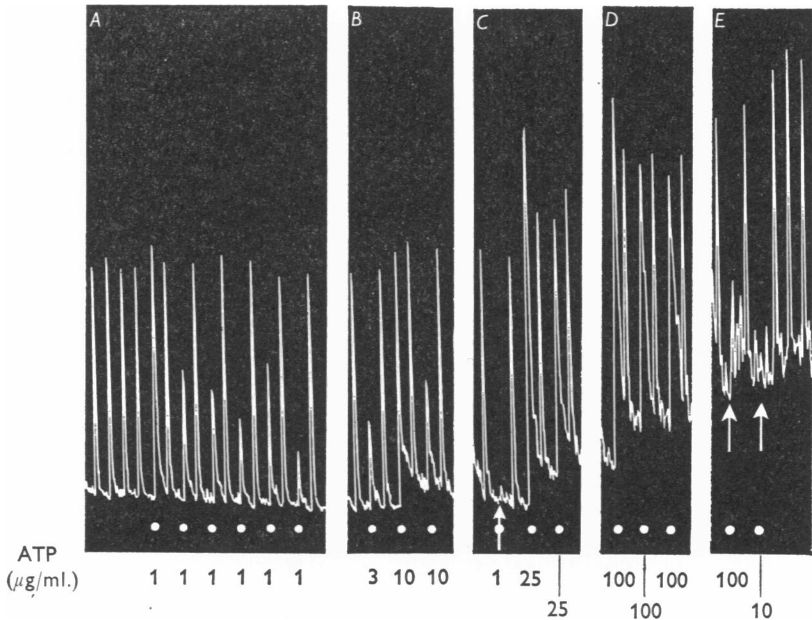

Fig. 7. The electrically induced twitch response remains unaffected by the tachyphylaxis and desensitization to ATP. Continuation of the experiment described in the legend of Fig. 6; records obtained on a slow drum. The preparation was stimulated at 1 min intervals, either with single 0·1 msec pulses or, at the white dots, with ATP left in the organ bath for 30 sec in the doses indicated; in panels *C* and *E*, if there was little or no response to the ATP, the injections have been marked by arrows above the dots. Just before panel *E*, the desensitization of the preparation was increased by a continuous exposure for 5 min to ATP, 100 μg/ml., and for a further 30 sec to 200 μg/ml. Details in text.

and $PGF_{2\alpha}$, was usually low and $PGF_{2\alpha}$ was weaker than PGE_2. With PGE_2 even at doses of 500 ng/ml. the contractions were sluggish and greatly delayed, only beginning 20–30 sec after the injections. The effect was long lasting and persisted for some 10 min after the PGE_2 was washed out.

The competitive antagonist SC-19220 (formula given in Methods) is known to abolish the action of PGE_1 and PGE_2 on other smooth muscle preparations (Sanner, 1969; Ambache *et al.* 1970). The limit of its water

solubility is said to be *ca.* 5 μg/ml. (J. H. Sanner, personal communication). In the present experiments, in concentrations of 1–5 μg/ml., it failed to block the PGE_2 response or to reduce the twitches elicited by single pulses. In another experiment the solid SC-19220 (10 μg/ml.) was added to the reservoir fluid. This reduced the response to PGE_2, 50 ng/ml., by 76·3 %; at the same time the responses to 1–5 pulses were only slightly diminished, by 21, 21, 23, 7 and 7·5 %, respectively.

Another prostaglandin-antagonist (Eakins & Karim, 1970), polyphloretin phosphate, 20 μg/ml., failed to block the effect of PGE_2; in higher concentrations it depressed the muscle and abolished the contractile response to histamine, thus losing its specificity.

Although the results with these antagonists have been somewhat inconclusive, nevertheless the low sensitivity of the detrusor muscle to prostaglandins, the long delay in the onset of these sluggish responses and their persistence after the PGE_2 was washed out, make it unlikely that the unknown transmitter is a prostaglandin.

Bladders from other species

Isolated bladder preparations from two other species, namely the cat and the rabbit, were also examined; confirmatory evidence for non-cholinergic post-ganglionic motor transmission was obtained in both.

The spontaneous motility was very pronounced in rabbit but weak or absent in cat bladders (as in the guinea-pig). In both species the twitches elicited by single 0·1 msec pulses and the larger responses to 2–5 pulses (10 Hz) remained unaffected by hexamethonium or pentolinium, 10^{-4} g/ml., and either totally unaffected or only slightly reduced by atropine, 10^{-6} g/ml. The atropine-resistant responses were not potentiated by eserine, $2 \cdot 5 \times 10^{-6}$ g/ml., administered for 20–30 min but were completely abolished by tetrodotoxin, 2×10^{-7} g/ml. Noradrenaline, 1 μg/ml., produced a marked relaxation of the preparations and a great reduction in the twitch responses.

DISCUSSION

These results dispose of two possible explanations for the atropine-resistance of electrically induced contractions in the bladder, mentioned in the Introduction, namely (*a*) that the 'muscarinic' ACh receptors in the detrusor muscle behave anomalously towards atropine; and (*b*) that these receptors are flooded by an excess of ACh liberated by the volley of nerve impulses, the excess overcoming the atropine block quantitatively. With the neuronal 'nicotinic' ACh receptors put out of action by the continuous presence of hexamethonium or pentolinium, atropine never failed to block the action of ACh on the 'muscarinic' receptors in the muscle, and the twitch-matching dose of ACh was raised by a factor of 1000–2500. At the

same time, whilst care had been taken, by the use of single pulses, to reduce to a minimum the liberation of transmitter at the nerve endings in order to avoid receptor flooding, there was little or no reduction in twitch height by atropine, even when present in concentrations bordering upon the toxicological. When atropine (10^{-7} g/ml. or less) did exert some inhibitory effect upon the twitch response, this was always partial. There was no further reduction of the atropine-resistant component in the twitch by a ten- or hundredfold increase in the atropine concentration; this is quite unlike the classical antagonism of ACh by atropine, which has a definite quantitative relationship.

Another explanation that can be put forward is the suggestion, made by Dale & Gaddum (1930), that neurogenic ACh is released in close proximity to the receptors and beyond the 'atropine-barrier'. Our negative results with morphine and with eserine would dispose of this possibility. Morphine can under certain conditions depress drastically the release of ACh from cholinergic nerve-endings (Schaumann, 1956, 1957; Paton, 1957; Beleslin, Polak & Sproull, 1965). In the guinea-pig ileum the cholinergic twitch elicited by single pulses is greatly reduced by morphine as a result of the diminished ACh release (Paton, 1957, 1963); but this effect of morphine is lost if the stimulation frequency exceeds 1 Hz. Since the bladder twitches were elicited by single pulses at frequencies well below that critical level, this essential condition for obtaining the inhibitory action of morphine was satisfied in the present experiments. Yet no inhibition whatsoever was recorded, even with 5–10 times the necessary concentration of morphine.

Faced with the problem of atropine-resistance in certain parasympathetic neuro-effector junctions, Dale & Gaddum (1930) considered that negative evidence with atropine was outweighed by positive evidence provided by potentiation with eserine. However, in the bladder the negative result with atropine is only reinforced by the lack of potentiation with eserine; this is all the more striking because the response was capable of considerable increase with 1–4 extra pulses. It is difficult to envisage cholinergic transmission of the twitches without transmitter destruction by cholinesterase(s).

Before considering Dale & Gaddum's hypothesis further, some recent histological data on the innervation of the bladder are relevant. El-Badawi & Schenk* (1966) noted in four species (cat, dog, rabbit and rat) that the

* The claim made by these authors that the post-ganglionic motor neurones in the bladder are cholinergic is based entirely upon the histochemical demonstration of acetylcholinesterase in these nerve fibres. This might, however, be due to the fact that the ganglion cells of these neurones are cholinoceptive and that cholinesterase is detected in the axons as well as the cell bodies because these axons are so short. Alternatively, this histochemical method may lack some specificity, or the enzyme detected by it might be subserving a function other than that of ACh hydrolysis.

'parasympathetic innervation is uniformly rich throughout the bladder, with a 1:1 nerve to muscle ratio'; the endings form a 'neuroterminal plexus which surrounds every smooth muscle cell in the bladder wall'. And with the electron microscope Caesar, Edwards & Ruska (1957) have found that in the mouse bladder 'each and every muscle cell shows a close relationship to the axon at a well defined locus.' This anatomical arrangement is entirely different from that prevailing in the longitudinal layer of the guinea-pig ileum, where there is no individual innervation of the muscle fibres (see Paton & Zar, 1968, p. 15), and neurogenic ACh, liberated at a distance, has to diffuse across to the muscle fibres just like administered ACh and is therefore blocked equally well by atropine, with extinction of the twitch response. However, this radical difference between bladder and ileum in their mode of innervation does not account for the observed difference in the atropine susceptibility of their twitches, because in the ciliary muscle and in the sphincter pupillae there is, as in the bladder, a profuse individual innervation of the muscle fibres by parasympathetic post-ganglionic motor neurones, but in the eye neurogenic ACh is easily and totally blocked in all species by small amounts of atropine, with resulting mydriasis and loss of accommodation. Hence, it is not the high density of innervation *per se* which is to be held responsible for the atropine-resistance of the twitches in the bladder.

Electron microscopy of this neuromuscular junction has failed to provide any histological basis for the concept that neurogenic ACh might here be released beyond the 'atropine-barrier' (the muscle cell surface). Caesar *et al.* (1957) state that the axons of the autonomic nerves in the bladder never penetrate the plasma membrane of the muscle cells but pass or intrude into muscle cell pockets. 'At such intimate appositions of axonal and muscle plasma membranes, no intervening basement membrane is visible and the interspace is generally 20 mμ.'

On Dale & Gaddum's (1930) hypothesis the individual innervation of the detrusor muscle fibres would make it necessary to postulate the presence of two types of ACh receptors on each muscle fibre, namely (1) a type that is easily blocked by atropine, accounting for the observed insensitivity to administered ACh and (2) a type that would remain unblocked by atropine, to account for the persisting twitch responses. Since atropine produced little or no reduction in the bladder twitch, the neurogenic ACh would have to excite mainly the 'synaptic', Type 2 and not the nearby 'extrasynaptic', Type 1 receptors on the same fibres. It would also be difficult to explain how it is possible for the unblocked 'synaptic', Type 2 receptors to be insensitive to administered ACh whilst remaining free to respond to neurogenic ACh and, above all, why eserine failed to potentiate the effect of neurogenic ACh at these unblocked receptors. These considerations

illustrate the theoretical difficulties raised by having to postulate the existence, side by side on one and the same muscle fibre, of atropine-sensitive and atropine-resistant ACh receptors.

The possibility was also considered, in the present experiments, that the ACh receptors in the muscle fibres might not be wholly 'muscarinic' in nature, but also 'nicotinic', as in parts of the oesophagus. This can be ruled out, because of the absence of antagonism to the twitches by dimethyltubocurarine, and also because of the lack of potentiation by eserine.

In the face of all the facts presented in this paper, taken together, it would be illogical to retain the concept that the motor neurones in the bladder are cholinergic. Moreover, since most of the other atropine-resistant parasympathetic nerve-effects have now received some satisfactory alternative explanation, there is good reason to doubt whether such a thing exists as an atropine-resistant 'muscarinic' receptor.

We wish to thank Mr J. Verney for valuable assistance and our colleague Dr G. C. R. Morris for much helpful discussion and for criticism of the manuscript.

REFERENCES

AMBACHE, N. (1955). The use and limitations of atropine for pharmacological studies on autonomic effectors. *Pharmac. Rev.* **7**, 467–494.

AMBACHE, N. & FREEMAN, M. ANNE (1968). Atropine-resistant longitudinal muscle spasms due to excitation of non-cholinergic neurones in Auerbach's plexus. *J. Physiol.* **199**, 705–727.

AMBACHE, N., VERNEY, J. & ZAR, M. ABOO (1970). Evidence for the release of two atropine-resistant spasmogens from Auerbach's plexus. *J. Physiol.* **207**, 761–782.

AMBACHE, N. & ZAR, M. ABOO (1970*a*). An inhibitory action of histamine on the guinea-pig ileum. *Br. J. Pharmac.* **38**, 229–240.

AMBACHE, N. & ZAR, M. ABOO (1970*b*). Non-cholinergic nature of the post-ganglionic motor neurones in guinea-pig bladder. *J. Physiol.* **209**, 10–12*P*.

BELESLIN, D., POLAK, R. L. & SPROULL, D. H. (1965). The release of acetylcholine into the cerebral subarachnoid space of anaesthetized cats. *J. Physiol.* **177**, 420–428.

BELL, P. M. G. (1968). A new stimulator. *Br. J. Pharmac. Chemother.* **32**, 435–436*P*.

CAESAR, R., EDWARDS, G. A. & RUSKA, H. (1957). Architecture and nerve supply of mammalian smooth muscle tissue. *J. biophys. biochem. Cytol.* **3**, 867–877.

CHESHER, G. B. & THORP, R. H. (1965). The atropine-resistance of the response to intrinsic nerve stimulation of the guinea-pig bladder. *Br. J. Pharmac.* **25**, 288–294.

DALE, H. H. & GADDUM, J. H. (1930). Reactions of denervated voluntary muscle, and their bearing on the mode of action of parasympathetic and related nerves. *J. Physiol.* **70**, 109–144.

EAKINS, K. E. & KARIM, S. M. M. (1970). Polyphloretin phosphate—a selective antagonist for prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$. *Life Sci. Oxford* **9**, 1–5.

EDGE, N. D. (1955). A contribution to the innervation of the urinary bladder of the cat. *J. Physiol.* **127**, 54–68.

EDVARDSEN, P. (1968). Nervous control of urinary bladder in cats. *Acta physiol. scand.* **72**, 234–247.

EL-BADAWI, A. & SCHENK, E. A. (1966). Dual innervation of the mammalian urinary bladder. A histochemical study of the distribution of cholinergic and adrenergic nerves. *Am. J. Anat.* **119**, 405–428.

ELLIOTT, T. R. (1905). The action of adrenalin. *J. Physiol.* **32**, 401–467.

ELLIOTT, T. R. (1907). The innervation of the bladder and urethra. *J. Physiol.* **35**, 367–445.

HENDERSON, V. E. & ROEPKE, M. H. (1934). The role of acetylcholine in bladder contractile mechanisms and in parasympathetic ganglia. *J. Pharmac. exp. Ther.* **51**, 97–111.

HENDERSON, V. E. & ROEPKE, M. H. (1935). The urinary bladder mechanisms. *J. Pharmac. exp. Ther.* **54**, 408–414.

HUKOVIĆ, S., RAND, M. J. & VANOV, S. (1965). Observations on an isolated, innervated preparation of rat urinary bladder. *Br. J. Pharmac. Chemother.* **24**, 178–188.

JÖNSSON, G. & ZEDERFELDT, B. (1957). A study of Cetiprin as a urologic drug. *Urol. internation.* **4**, 293–305.

KAVANAGH, L. (1962). A light writing-point pivoted on watch-bearings for frontal levers. *J. Physiol.* **163**, 1–2*P*.

LANGLEY, J. N. & ANDERSON, H. K. (1895). The innervation of the pelvic and adjoining viscera. Part II. The bladder. *J. Physiol.* **19**, 71–139.

PATON, W. D. M. (1955). The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol.* **127**, 40–41*P*.

PATON, W. D. M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.* **12**, 119–127.

PATON, W. D. M. (1963). Cholinergic transmission and acetylcholine output. *Can. J. Biochem. Physiol.* **41**, 2637–2653.

PATON, W. D. M. & ZAR, M. ABOO (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J. Physiol.* **194**, 13–33.

SANNER, J. H. (1969). Antagonism of prostaglandin E_2 by 1-acetyl-2-(8-chloro-10,11-dihydrodibenz [b, f] [1, 4]oxazepine-10-carbonyl) hydrazine (SC-19220). *Archs int. Pharmacodyn. Thér.* **180**, 46–56.

SCHAUMANN, W. (1956). Influence of atropine and morphine on the liberation of acetylcholine from the guinea-pig's intestine. *Nature, Lond.* **178**, 1121–1122.

SCHAUMANN, W. (1957). Inhibition by morphine of the release of acetylcholine from the intestine of the guinea-pig. *Br. J. Pharmac. Chemother.* **12**, 115–118.

URSILLO, R. C. (1961). Investigation of certain aspects of atropine-resistant nerve effects. *J. Pharmac. exp. Ther.* **131**, 231–236.

URSILLO, R. C. & CLARK, B. B. (1956). The action of atropine on the urinary bladder of the dog and on the isolated nerve-bladder strip preparation of the rabbit. *J. Pharmac. exp. Ther.* **118**, 338–347.

VANOV, S. (1965). Responses of the rat urinary bladder *in situ* to drugs and to nerve stimulation. *Br. J. Pharmac. Chemother.* **24**, 591–600.