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ATROPINE RESISTANCE AND MUSCARINIC RECEPTORS IN THE RAT URINARY BLADDER

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1 The action of an anticholinesterase and an antimuscarinic drug upon nerve-induced contractions of the rat urinary bladder were examined during transmural stimulation at 20 Hz. Responses were graded in magnitude by limiting the duration of the stimulus trains.

2 Responses of low magnitude produced by short stimulus trains were unchanged by atropine; however, maximal responses resulting from long stimulus trains were diminished in magnitude and shortened in duration.

3 Responses of small magnitude elicited by short stimulus trains involve muscarinic receptors in close proximity to the neuroeffector junction and are resistant to atropine.

4 Maximal responses elicited by long stimulus trains involve 'junctional' muscarinic receptors as well as receptors located at the periphery of the junction; the 'extrajunctional' receptors are blocked by atropine.

5 Responses of low magnitude produced by short stimulus trains were unaffected by echothiophate; however, the duration of maximal responses resulting from the long stimulus trains was extended.

6 The inhibition of cholinesterase did not increase the occupation of muscarinic receptors by the transmitter; however, after large quantities of transmitter were released by the long stimulus trains the association between the receptors and acetylcholine was prolonged.

Introduction

The motor innervation of the mammalian urinary bladder is usually represented as cholinergic even though nerve-induced responses of this organ are not completely prevented by conventional antimuscarinic agents such as atropine or scopolamine (Ursillo & Clark, 1956; Carpenter, 1963; Vanov, 1965; Ambache & Zar, 1970). Although these drugs do reduce or block bladder contractions elicited by carbachol or by acetylcholine itself, some authors have proposed that the motor nerve terminals of the urinary bladder are non-cholinergic (Ambache & Zar, 1970; Dumsday, 1971; Burnstock, Dumsaday & Smythe, 1972). The atropine resistance of the urinary bladder may result from a close association between the nerve terminals of this organ and the muscle cell membrane; the junction or point of contact between the post-ganglionic nerves and the smooth muscle membrane may not be readily accessible to antimuscarinic agents.

There is strong evidence that acetylcholine is a transmitter in the rat urinary bladder: (1) nerve-induced responses of the bladder are potentiated by physostigmine (Ursillo & Clark, 1956; Carpenter, 1963; Chesler & Thorp, 1965; Huković, Rand & Vanov, 1965) (2) acetylcholine accumulates in the bathing solution *in vitro* in proportion to the stimulus rate (Carpenter & Rand, 1965; Chesler, 1967); (3) the responses of the rat bladder and the release of acetylcholine during stimulation are greatly reduced following pretreatment of the rats with *Cl. botulinum* toxin (Carpenter, 1967).

This study is concerned with the action of ecothiophate and atropine on nerve-induced responses of the urinary bladder *in vitro*. Its purpose is to show that those junctional events elicited by the small amount of transmitter released during brief stimulation were not affected by substances which block muscarinic receptors or by substances which extend the life of the transmitter. However, during prolonged stimulation which elicits more powerful contractions of the muscle, events at the junction were very significantly affected by these drugs; the contractile responses were more susceptible to the blocking actions of an antimuscarinic agent such as atropine and to a drug such as echothiophate which prolongs the action of the transmitter.

Methods

Phasic contractions of the rat bladder were elicited isometrically in a constant temperature water bath

(Carpenter, 1963). The organ was stimulated by single or repetitive electrical pulses of 1 ms duration. A constant stimulating current of 25 mA was passed across the bladder wall between two coaxial electrodes composed of platinum wire. One electrode located in the bladder lumen projected through the cannula on which the organ itself was secured. The other electrode was fixed in the organ bath and surrounded the preparation in the bathing solution. The cannula was connected to a pressure transducer by flexible tubing. Care was taken to displace any air bubbles before the experiment started. The magnitude of a given response was obtained from recordings of the pressure that developed while the stimuli were applied. Each response was recorded on an ink-writing oscillograph and was photographed on high speed film from a cathode ray oscilloscope (CRO).

The excitation of bladder smooth muscle by transmural stimuli is indirect and mediated by acetylcholine (ACh) released from postganglionic nerve (Carpenter & Rand, 1965); hexamethonium or mecamylamine did not affect the responses while tetrodotoxin and certain other local anaesthetics prevented them.

Throughout the experiments the stimuli were applied as single pulses or as trains of pulses at a rate of 20 Hz. Responses of four different magnitudes between 20 and 160 cmH_2O were produced by controlling the number of pulses; the mechanical response of a bladder preparation was graded by limiting the duration of the trains to 0.25, 0.5 and 5.0 seconds. The stimulus trains were applied intermittently and it was found that a 3 min period was adequate for recovery as shown by the consistent reproducibility of the responses for as long as 90 minutes.

There are several mechanical factors which can influence the contractility of the rat bladder. Both the velocity and the magnitude of a response are related to the initial length of the muscle fibres and to their state of elasticity. Throughout its physiological capacity as a storage organ the muscle in the rat bladder displays a length-tension relation similar to cardiac or somatic muscle. In these experiments the volume in the organ was maintained at 0.15 ml which is much less than the volume it contains at the time of voiding. Nevertheless, under these conditions the muscle fibres develop their maximal force during an isometric contraction; this indeed suggests that the muscle fibres were set at their optimal length. Moreover, since the volume was constant during a contraction, the force developed by the response can be calculated from the Laplace relation: $T = P.r^2$, where T is the force or tension, P is the pressure and r is the radius of the bladder which may be assumed to be spherical.

Echothiophate (0.3 mg/kg) or atropine sulphate (2.5 mg/kg) were administered to adult male rats weighing between 400 and 450 grams. Within 30 min

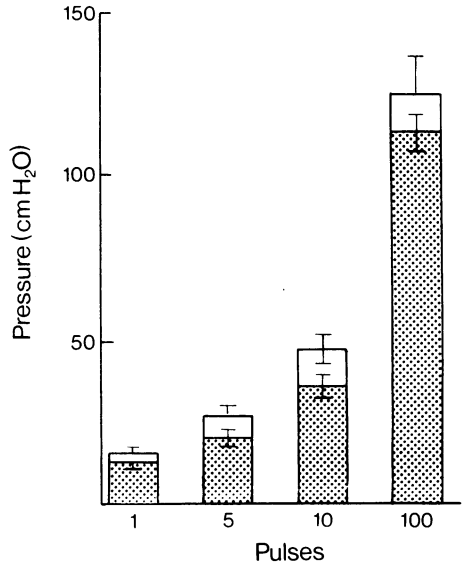

Figure 1 The pressure developed by rat urinary bladder *in vitro* during stimulation by electric pulses. The mean response to a single stimulus is shown by the 1st column and the mean responses to 5, 10 and 100 pulses applied at a stimulus rate of 20 Hz are shown by the 2nd, 3rd and 4th columns respectively. Ordinate scale: pressure in cmH_2O. The number of pulses that were applied appears at the base of each column. Preparations taken from untreated control rats are represented by the open area ($n = 12$) while the preparations removed from echothiophate iodide-treated (0.3 mg/kg) rats ($n = 12$) are represented by the stippled area. The s.e. is indicated by the vertical lines.

the animals were killed, the bladder removed and placed in a bathing solution of the following composition (mM): NaCl 145, KCl 2.7, $CaCl_2$ 2, $MgCl_2$ 0.25, MOPS (morpholino-propane-sulphonic acid) buffer adjusted to pH 7.3, 10 and glucose 10. The dose of echothiophate that was administered amounted to 10 times the rabbit LD_{50} (Koelle & Steiner, 1956) and its action on the bladder did not diminish while the experiments were carried out. On the other hand, bladder preparations taken from the atropine-treated animals were suspended in the bathing solution containing atropine sulphate 20 μM.

Results

Bladder contraction in control preparations produced by single stimulus pulses

Phasic contractions of the rat bladder produced by single stimuli developed only 1/10 as much force as

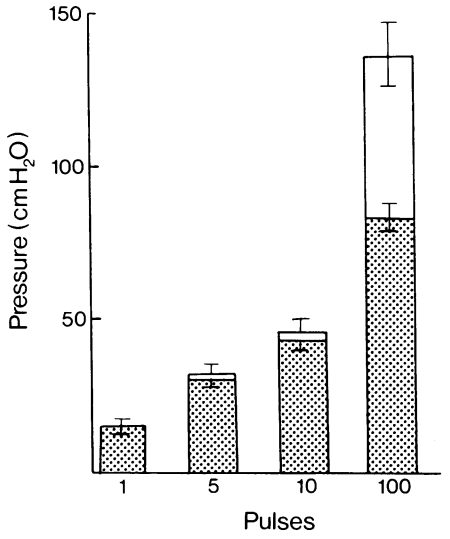

Figure 2 The pressure developed by the rat urinary bladder *in vitro*. Preparations taken from untreated control rats are represented by the open area ($n=13$) while the preparations removed from atropine-treated (2.0 mg/kg) rats ($n=10$) are represented by the stippled area. The s.e. is indicated by the vertical lines. Other details as for Figure 1.

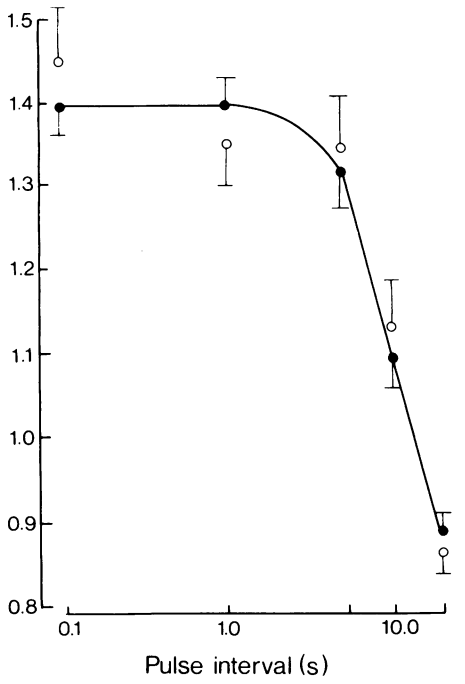

Figure 3 The relative magnitude of bladder responses elicited by 2 stimulus pulses applied at different intervals. Ordinate scale: response to dual pulses/responses to single pulse, expressed as the mean. Abscissa scale: interval between pulses in seconds. (O) Preparations taken from untreated rats ($n=6$); (●) preparations taken from rats treated with 0.3 mg/kg echothiophate idodide ($n=5$). The s.e. is indicated by the vertical lines.

those elicited by the 20 Hz 5 s stimulus trains. After only one pulse was applied to the coaxial electrodes, contraction between 12 and 18 cmH_20 were produced (Figures 1 and 2). In 15 observations the mean pressure developed by the preparations amounted to 16 ± 2. Within 0.2 s (latency) after the pulse was applied the peak pressure developed some 2 s later and subsided to the original resting pressure in approximately 8 s (relaxation time).

Bladder contractions produced by dual stimulus pulses

When a second pulse was applied after the initial pulse a further increment of pressure was produced by the bladder muscle. This increment is expressed in Figure 3, as the mean of the ratios of the response following two pulses to the response after only pulse. If the ratio is greater than one, the contraction elicited by a second stimulus summated mechanically with the initial contraction. The time course of summation was examined at intervals of 0.1, 1.0, 5, 10 and 20 s, and appear as open circles in Figure 3. It will be noted that the response to the second pulse declined progressively when the interval was longer than 5 seconds. Nevertheless, since the ratio at 10 s was greater than one, summation could occur at this interval. However, at 20 s the ratio was always less than one.

Bladder contractions after single and dual stimuli of preparation taken from echothiophate-treated animals

In the group that had been given the cholinesterase inhibitor and had been stimulated by single pulses, the latency, the response time, the relaxation time and the mean of the responses themselves were not different from the control group. In Figure 1 the mean response of the treated group is shown by the shaded area in the first column.

If phasic contractions of the bladder are mediated by ACh, the transmitter released during a response would most probably be hydrolyzed prior to relaxation. Indeed, if acetylcholinesterase restricts the accumulation of the transmitter, the response of the treated preparations to dual stimuli would be different from the control; if the action of the transmitter which had been released after the first stimulus had been prolonged by echothiophate the responses elicited by the second stimulus would have been displaced above the control group. However, the data obtained from

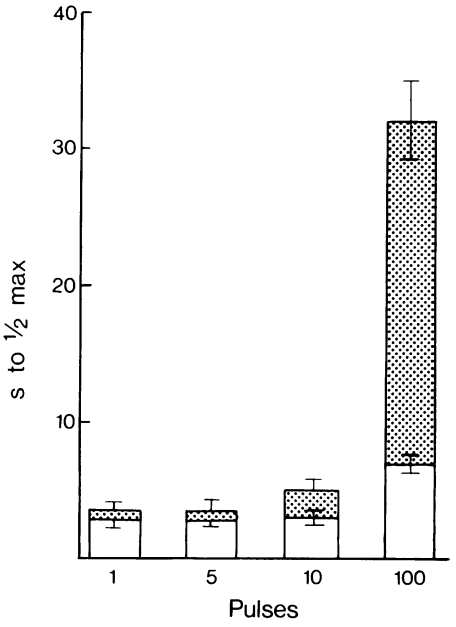

Figure 4 The duration of contractile responses of the rat urinary bladder elicited by a single stimulus pulse (1st column) and by stimulus trains of 5, 10 and 100 pulses (2nd, 3rd and 4th columns). The mean of 8 non-treated control animals is represented by the open area of each column while the 10 rats treated with echothiophate iodide (0.3 mg/kg) is shown by the stippled area. The s.e. is indicated by the vertical lines.

control and treated groups shown in Figure 3 are not significantly different.

Bladder contractions produced by stimulus trains

Repetitive stimulation of the bladder elicits responses which depend upon the stimulating frequency and the number of stimuli that are applied. In the range of 1 to 20 Hz the magnitude of a contraction is related to the stimulus rate. At 20 Hz, around 100 pulses are usually necessary for the bladder to produce a maximal response. The mean responses of the two groups, shown in the last column of Figures 1 and 2, amounted to 125 ± 12 and 136 ± 10 cmH_20 respectively. The third columns of Figures 1 and 2 indicate the mean responses which were produced when only 10 pulses were applied and amounted to roughly one-third of maximal or 47 ± 5 and 46 ± 3 cmH_20 respectively. After 5 stimulus pulses were applied the mean contractile response of the two groups, which appear in the second columns, were 37 ± 1 and 32 ± 1 cmH_20.

Bladder contractions produced by stimulus trains in treated preparations

Echothiophate. Echothiophate did not increase the magnitude of the contractile responses that resulted from multiple stimuli. In fact, the stimulus trains elicited somewhat less of a pressure rise in the treated group than in the untreated controls. In Figure 1 the mean responses, shown in the second and third column and elicited by 5 and 10 stimulus pulses respectively, were significantly less in the group treated with echothiophate. Although the mean of the responses elicited by the 5 s stimulus train was less than control, the difference between control and treated groups was not significant (fourth column, Figure 1).

Despite the failure of echothiophate to enhance contractile responses to single, dual or to trains of stimuli, acetylcholinesterase nevertheless, does appear to limit the transmitter action of acetylcholine in the bladder. The duration of a response following single stimuli and following the three stimulus trains was measured for control and the echothiophate-treated groups, In Figure 4 this is expressed as the average number of seconds required for the pressure to diminish to one-half the value of the peak response (relaxation time). After single stimuli and after trains of 0.25 and 0.5 s, relaxation occurred within 3 s in the control untreated group; the height of the first three columns is the same. After the 5 s trains the mean relaxation time was 7 seconds. There is little difference between treated and control groups in the first three columns; the mean relaxation time following short periods of stimulation was not significantly prolonged by the inhibitor. However, the relaxation time following the 5 s stimulus train was extended by 5-fold from control to nearly 35 (last column, Figure 4).

Recordings of the contractile responses by a control bladder preparation to the various stimulus trains are shown in Figure 5. It should be compared with the recordings of the responses to the same stimulus trains of a treated preparation which is shown in Figure 6. These records indicate further, that the most significant prolongation of the responses occurred following the longest stimulus train. Only in the latter instance was the response time of the treated preparation significantly extended from the control.

Atropine. Atropine (20 μM) did not exert a significant action on the rat bladder if the responses were elicited by the short stimulus trains. In the third column of Figure 2, the 20 Hz stimulus train was applied for only 0.5 s; the mean response of the control and of the treated group were not significantly different. The mean responses shown in the first and second column also show that a bladder contraction resulting from either a single pulse or a train applied for only 0.25 s was not changed by atropine. Accordingly, any response elicited by one, five or as

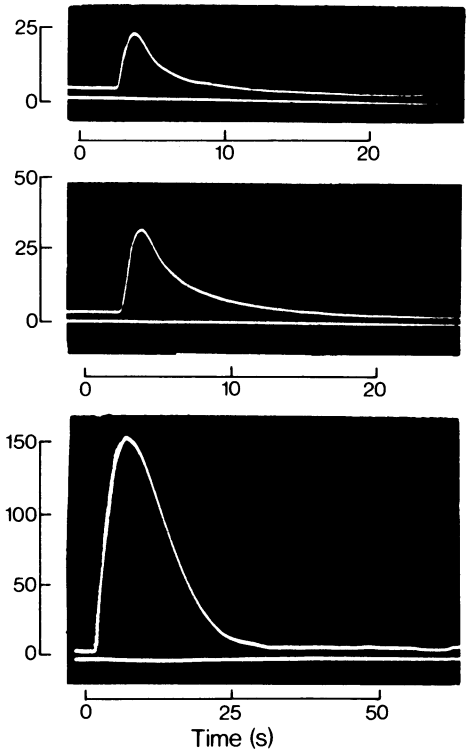

Figure 5 Recordings taken from a CRO of the contractile responses of a control untreated rat urinary bladder after 20 Hz stimulus trains were applied for 0.25 (upper), 0.5 (middle), and 5.0 s (lower). The upper and middle traces were made at twice the gain and 2.5 times the sweep speed as the lower trace. The appropriate pressure calibrations appear in the left margins while the time base is located below the recordings.

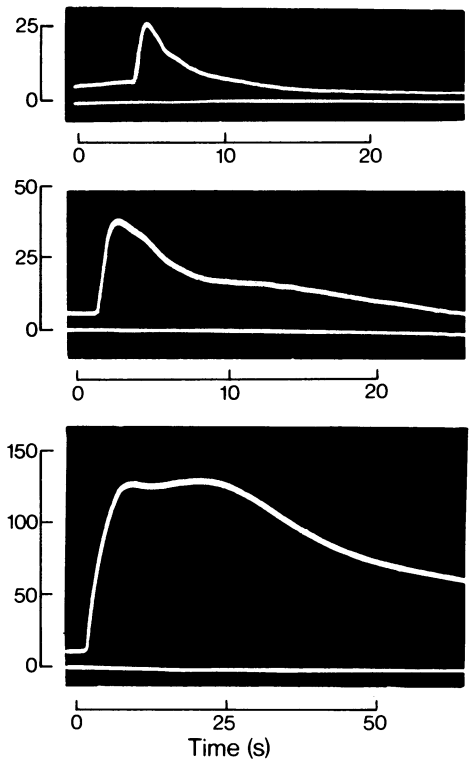

Figure 6 Recordings taken from a CRO of the contractile responses of a bladder preparation removed from a rat treated with echothiophate (0.3 mg/kg). Stimulus trains at 20 Hz were applied for 0.25 (upper), 0.5 (middle), and 5.0 s (lower). The upper and middle traces were made at twice the gain and 2.5 times the sweep speed as the lower trace. The appropriate pressure calibrations appear in the left margins while the time base is located below the recordings.

many as 10 pulses was not changed significantly by this drug even though it completely prevented the action of exogenous ACh (50 μM) and carbachol (25 μM). However, there was one further effect caused by the atropine which suggested that there was a partial blockade of muscarinic receptors which were involved in the nerve-induced responses of the control preparations. This was evident by the very significant difference (40%) between the mean responses of the control and the atropine-treated preparations resulting from stimulation by the 5 s stimulus trains (last column, Figure 2).

The contractile force of the rat bladder developed quite rapidly during repetitive stimulation at 20 Hz and the intraluminal pressure continued to increase throughout the 5 s stimulus train (Figure 7). Atropine (20 μM) did not interfere with the response which developed initially; the rate at which the pressure developed during the first two seconds appeared to be identical in the control and in the treated animals. In Figure 7 are shown two recordings of the contractile response elicited by a 5 s, 20 Hz train; the upper trace in each case was obtained from a control preparation; the lower trace was the result of the treatment with atropine. In 25 experiments the mean response of a non-treated preparation was always greater than the response following atropine. Nevertheless, the rising phase of the contractions; which of course represented the initial response to the stimulus train, was the same in both cases; treated bladders maintained the same rate of contraction during the initial 0.5 s of the response as the controls. In a number of others the initial rise time appeared to be unchanged from the control for as long as one second. The blocking action

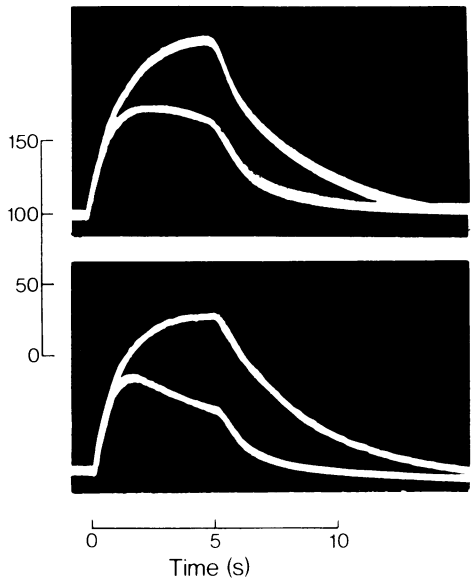

Figure 7 Recordings taken from a CRO of the contractile responses of the bladder from two rats, showing the response of the control untreated rat urinary bladders (upper trace in each case) and response after they were treated with atropine sulphate (20 μM) (lower trace). The pressure calibration appears in the left margins and the time base is located below the recordings.

of atropine was only manifested later during the peak of the contraction in treated preparations. Indeed, the most striking effect of the atropine appeared during the final 3 s of the response itself; the muscle appeared to be undergoing relaxation during the latter part of the stimulation period (Figure 7). In contrast, the control preparations either maintained a constant pressure throughout the stimulation period or would continue to rise significantly while the stimulus train was being applied.

Discussion

During a contractile response the pressure in the bladder is elevated in proportion to the number of receptors occupied by the transmitter. Transmural stimulation in the range 1–20 Hz elicits responses in proportion to both stimulus rate and the amount of transmitter that is released during the stimulus period. When stimuli are applied at 20 Hz for a 5 s period much more of the transmitter diffuses from the junction than would occur after single, dual or short trains of stimuli. Accordingly, there would be a greater involvement of muscarinic receptors that are located some distance from the junction since the actual contact between the nerve terminal and the muscle fibres themselves is probably quite small.

Echothiophate and receptor-transmitter combination

Acetylcholinesterase apparently plays no major role in limiting the combination of transmitter with the muscarinic receptors; there was no difference between the echothiophate-treated and the control groups with respect to the peak pressure responses, as shown in Figure 1. The difference between these two groups was in the duration of the responses following the long stimulus trains. It would thus appear that transmitter action on some but not all receptors was prolonged by AChE inhibition. Since the response to single, dual (Figure 3) and the short stimulus trains was not affected by echothiophate, while maximal responses were prolonged by it (Figure 4 and 6), it seems reasonable to suppose that echothiophate either cannot gain access to the space close to the junction or that hydrolysis of the transmitter occurs only at some distance from the junction.

Atropine and receptor-transmitter combination

The recordings of Figure 7 indicate that the diffusion of ACh from the junction during a long stimulus train must be quite extensive; the recruitment of all muscarinic receptors was completed after 5 s of stimulation. However, much less time was needed for recruitment during single, dual and the short stimulus trains. In the latter cases receptor-transmitter combination may have occurred much more rapidly with receptors close to the junction. Of course during the diffusion of transmitter from the junction the concentration of transmitter would decrease exponentially with distance.

Atropine was not an effective antagonist of the transmitter when single, dual or short stimulus trains were employed (Figure 2). If the concentration of ACh is very high in the junctional area the receptors would be more resistant to blockade by atropine. Moreover, the early phase of the response shown in Figure 7 was not impaired by atropine. Only during the late phase of the response was any antagonism between atropine and the transmitter clearly demonstrated. The late phase of a prolonged response would involve more distant receptors. These 'extrajunctional receptors' would be exposed to lower concentrations of transmitter as would be anticipated by diffusion and they would be more vulnerable to blockade. Phasic contractions of the guinea-pig bladder elicited by 10 Hz trains of 2–5 pulses were similarly resistant to large doses of atropine (Ambache & Zar, 1970) while contractions produced by single pulses were not enhanced by physostigmine. Accordingly, in this species as well, anticholinesterase and antimuscarinic

agents were without effect on bladder responses of small magnitudes.

Junctional and extrajunctional receptors in the rat bladder

There are major differences between a response produced *in vitro* by a cholinomimetic such as carbachol or acetylcholine and a response elicited by stimulation with electric pulses. Atropine, at a concentration of 1 μM for example, will compete with the agonists in the bathing solution causing a shift in the dose-response curve. A larger concentration of atropine, 20 μM, will eliminate the response to the cholinomimetics altogether. In this concentration atropine will block only 40% of the bladder response to the long stimulus trains. However, a maximal response which results from the long stimulus train is some 50% higher than the maximal response to high concentrations of the cholinimimetics (Carpenter & Rand, 1965; Carpenter, 1967). This suggests that the entire population of muscarinic receptors cannot combine with the cholinomimetic in the bathing solution; the junctional receptors may not combine with the agonist because of limitations of access. The difference between the neuroeffector junction of the bladder and other cholinergic junctions with respect to 'atropine resistance' may be partially resolved by the division of the receptor population into two spatially distinct sites.

Non-cholinergic transmission in the rat urinary bladder

These results offer no support for a non-cholinergic transmitter in the rat urinary bladder. Echothiophate and atropine modified the responses of this organ in a way that could be predicted by the specificity of echothiophate for the cholinesterase enzyme and the specificity of atropine for muscarinic receptors. These actions of echothiophate and of atropine were manifested only during maximal responses. Under these conditions transmission would be accompanied by the release of a larger amount of transmitter than during stimulation by single, dual or short trains of stimuli. If two transmitters were involved a small submaximal response elicited by a few impulses would have to be mediated by a non-cholinergic transmitter while the maximal responses elicited by the long stimulus trains would somehow have to involve acetylcholine.

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