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Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and α,β-methylene ATP

A.F. Brading & J.H. Williams

University Department of Pharmacology, South Parks Road, Oxford OX1 3QT

1 Strength-duration curves for threshold mechanical responses to single transmural stimuli were identical for rat and guinea-pig detrusor. In both species atropine had no effect on the curves, but the curves were shifted to the right by nerve blockade with tetrodotoxin (TTX), and by blockade of P_2-purinoceptors with α,β-methylene ATP (α,β-MeATP).

2 With short duration pulses of 50 V and less, the responses were nerve-mediated. Increase in either the strength or duration of the stimulus caused direct muscle stimulation, resistant to blockade with atropine, TTX and α-β-MeATP.

3 The shape of the contractile response to a single nerve stimulus varied from tissue to tissue. The responses could be mono-, bi-, or multiphasic. Bi- or multiphasic responses were normally seen in tissues which were spontaneously active. The multiphasic nature of the response was enhanced by factors which increased the excitability of the cells and was reduced by factors which decreased the excitability.

4 The frequency-response curves in the rat are similar to those previously obtained in the guinea-pig. Atropine suppresses the high frequency response by 25%, with little effect at low frequencies, whereas desensitization of P_2-purinoceptors with α,β-MeATP suppresses the responses maximally at low frequencies but still by 75% at high frequencies. A combination of both drugs eliminates the nerve-mediated responses.

5 It is concluded that the response to a single nerve stimulus is mediated by a non-cholinergic transmitter, through activation of P_2-purinoceptors. The possibility that simultaneous release of acetylcholine can modify the excitability of cells and thus the configuration of the response to a single stimulus is discussed.

Introduction

Contractile activity can be initiated in the smooth muscle of the urinary bladders of most mammals by stimulation of the parasympathetic nerves, or by field stimulation of intrinsic excitatory nerves in smooth muscle strips. The contractile response has, however, long been known to be partially resistant to blockade with atropine (Langley & Anderson, 1985; Henderson & Roepke, 1934; Ursillo & Clark, 1956). In the guinea-pig, Burnstock and his colleagues have provided evidence that the non-cholinergic excitatory transmitter is adenosine 5'-triphosphate (ATP) (Burnstock *et al.*, 1978a,b), and it has also been shown (Kasakov & Burnstock, 1983; Mostwin, 1986; Brading, 1987) that the contractile response of guinea-pig detrusor strips to transmural stimulation can be blocked by a combination of atropine and desensitization of the P_2-purinoceptors with α,β-methylene ATP (α,β-MeATP). Atropine has little effect on the contractile response at low frequencies of stimulation, whereas desensitization of the P_2-purinoceptors is most effective at low frequencies of stimulation.

Electrophysiological studies on rabbit and guinea-pig detrusor (Hoyle & Burnstock, 1985; Fujii, 1988) have demonstrated that excitatory junction potentials can be elicited by single transmural nerve stimuli, and that they are unaffected by atropine, and blocked in the presence of α,β-MeATP. Application of ATP causes depolarization of the cell membranes, whereas acetylcholine contracts the tissues at concentrations that have little effect on the membrane potential, and only a modest effect on spike frequency. It seems likely in the guinea-pig, that the contractile response to a single stimulus of the intrinsic nerves is largely due to the excitatory junction potential initiating synchronous action potentials in the tissue, whereas the cholinergic response is largely initiated through pharmaco-mechanical coupling, and is more effective when acetylcholine is released by repetitive stimulation.

Recently, studies in rat detrusor strips have suggested that the response to a single nerve stimulus may often be biphasic, the second phase being preferentially blocked by atropine at a concentration of 3×10^{-6} M (Maggi *et al.*, 1985; Bhat *et al.*, 1989). Bhat *et al.* (1989) also demonstrated that the second phase is preferentially blocked by lowering extracellular Ca and by Ca channel blockers, findings that would not be expected for a drug acting primarily through pharmaco-mechanical coupling. In fact the authors showed that the response to bath-applied acetylcholine was less susceptible to Ca antagonists than the atropine-sensitive component of the neurogenic response.

To clarify this, we have compared the contractile responses of strips of rat and guinea-pig smooth muscle, recording the effects of atropine, α,β-MeATP and tetrodotoxin (TTX) on strength-duration curves and frequency-response curves. We have also examined the shape of the contractile response to single nerve stimuli. Our results suggest that the behaviour in both species is very similar, and that the slower components of the contractile response are more dependent on the level of excitability of the tissue than they are on activation of muscarinic receptors.

Methods

Rats and guinea-pigs of either sex were stunned by a blow to the head, and bled. The urinary bladder was removed, and opened by a ventral incision from the urethra to the dome. With the aid of a dissecting microscope, the mucosa was removed from the smooth muscle and strips of detrusor of about 1 mm by 10 mm containing parallel muscle bundles were prepared. The strips were mounted in small chambers (0.2 ml capacity, Brading & Sibley, 1983) and continuously superfused with Krebs solution (35–37°C) at a flow rate of approximately 1 ml min^{-1}. Recessed platinum ring electrodes,

1 cm apart, were used for stimulation. The apparatus allowed six strips to be studied simultaneously. Tension was measured isometrically on Pioden transducers and the output fed to a six channel Watanabe pen recorder. The strips were initially placed under 0.5 g resting tension, and allowed to equilibrate for at least an hour.

Krebs solution contained (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.2, NaH_2PO_4 1, $CaCl_2$ 2.5 and glucose 11. Solutions were equilibrated with 97% O_2, 3% CO_2. Drugs used were atropine sulphate and carbachol, (B.D.H.), TTX and α,β-MeATP (Sigma).

Strength-duration curves were constructed by recording the voltage needed at each duration of stimulus to produce a just noticeable contraction of the tissue. After obtaining a control curve, the tissues were incubated in one of the three drugs, TTX, atropine or α,β-MeATP for at least 10 min and the procedure repeated in the continuous presence of the drug. For the frequency-response curves, a pulse duration of 0.05 ms and strength of 50 V was chosen, and the tissues were stimulated with 5 s trains of pulses at increasing frequencies, leaving 10 min between each stimulation. The responses were expressed as a percentage of a control response to a 10 s exposure to 10^{-4} M carbachol.

Results are expressed as the mean ± s.e.mean.

Results

Strength-duration curves

The strength-duration curves were remarkably similar in the two species, as illustrated in Figures 1 and 2. In the presence of 5×10^{-7} M atropine, a concentration which virtually abolished the tissue response to 10^{-4} M carbachol, the strength-duration curves were unaltered. In the presence of 1.6×10^{-6} M TTX the curves were shifted upwards and to the right.

Preliminary experiments in which muscle strips were continuously perfused with Krebs solution containing 10^{-5} M α,β-MeATP revealed that this concentration of drug did not abolish the contractile response of the muscle strips to short applications of 10^{-4} M α,β-MeATP, therefore a concentration of 10^{-4} M was used, which completely desensitized the P_2-purinoceptors. On application of the drug in this dosage, the tissues contracted, and then relaxed back to the baseline and did not respond to a further 10 s application of 10^{-2} M α,β-MeATP. The strength-duration curves in the presence of this drug were also shifted upwards and to the right. In the guinea-pig (Figure 1), α,β-MeATP shifted the curves significantly more than did TTX, suggesting that either the drug may have some additional effect on the excitability of the smooth muscle, or that in the presence of TTX, high voltages can release some transmitters from the nerve terminals. In the rat, the number of experiments was too small to show any significant difference between the effects of TTX and α,β-MeATP.

The response to single shocks

The shape of the contractile response to a single shock varied considerably from preparation to preparation, and also depended on the parameters of the stimulus used and the length of time since the preparation had been set up.

Correlation with spontaneous activity One of the most important determinants of the shape of the response seemed to be the degree of spontaneous activity shown by the preparation. In the majority of cases where there was no spontaneous activity, the contractile response elicited by a single stimulus was simple in shape, and showed only a single component. When spontaneous activity was seen, the response to a single stimulus was normally bi- or multiphasic, although in the guinea-pig monophasic responses were seen in some tissues

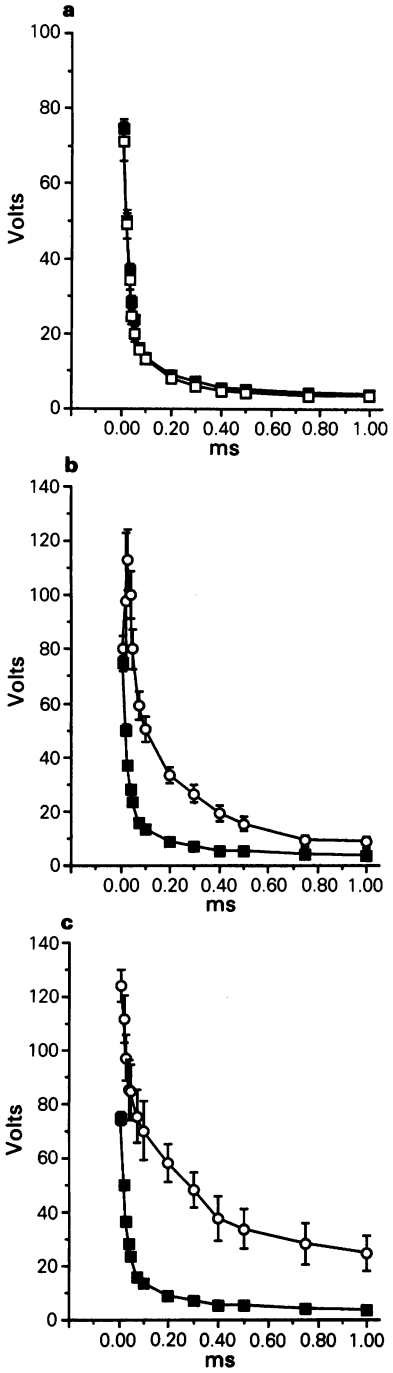

Figure 1 Strength-duration curves for threshold mechanical responses to single transmural electrical stimuli in strips of guinea-pig detrusor. In each panel (■) indicate control responses. (a) The effect of muscarinic receptor blockade with 5×10^{-7} M atropine. (b) The effect of blocking action potentials in the nerves with 1.6×10^{-6} M tetrodotoxin (TTX). (c) The effect of desensitization of P_2-purinoceptors with 10^{-4} M α,β-methylene ATP (α,β-MeATP). Note the lack of effect of atropine, and the fact that the curves are shifted further by α,β-MeATP than by TTX. Points show mean, s.e.mean (n = 11–34) shown by vertical bars where larger than the symbols.

that had relatively low frequency spontaneous activity. Since the rat tissues showed spontaneous activity more frequently than the guinea-pig tissues (this occurred in 90% of rat strips and only 60% of guinea-pig strips, n = 21 and 15 respectively), bi- and multiphasic responses were more commonly seen in rat strips. When spontaneous contractions occurred in the guinea-pig, they were always of a lower frequency than in the rat, and often disappeared during the course of the experi-

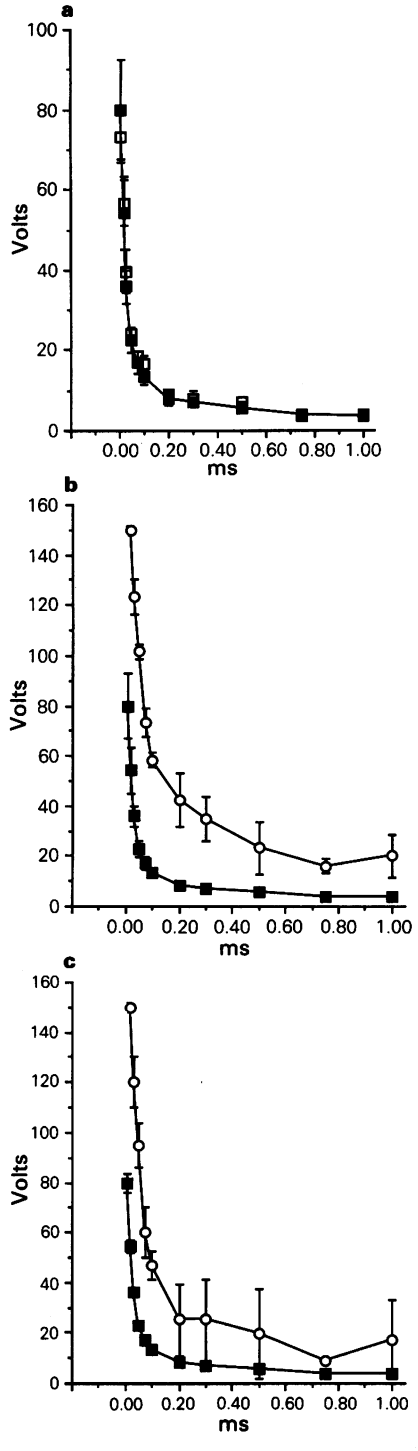

Figure 2 Strength-duration curves for threshold mechanical responses to single transmural electrical stimuli in strips of rat detrusor. In each panel (■) indicate control responses. (a) The effect of muscarinic receptor blockade with 5×10^{-7} M atropine. (b) The effect of blocking action potentials in the nerves with 1.6×10^{-6} M tetrodotoxin. (c) The effect of desensitization of P_2-purinoceptors with 10^{-4} M α,β-methylene ATP. Note the lack of effect of atropine. Points show mean, s.e.mean ($n = 2$–6) shown by vertical bars where larger than the symbols.

ment. In this case, the response to a single stimulus usually changed from being bi- or multiphasic to being monophasic.

Effect of alteration of stimulus parameters The effect on the magnitude of the contractile response of increasing the pulse duration at constant voltage of 50 V and increasing voltage at a constant pulse duration of 0.05 ms, is shown in Figure 3. With a 50 V stimulus, there was no response at a pulse width

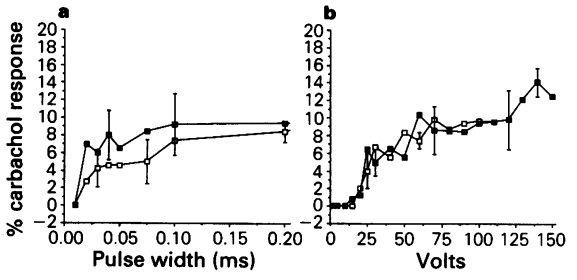

Figure 3 The effect on the contractile response of guinea-pig (■) and rat (□) detrusor strips of (a) increasing the pulse width of single 50 V transmural stimuli, and (b) increasing the stimulus strength of single 0.05 ms stimuli. Points are means of three tissues. Representative s.e.mean are shown.

of 0.01 ms. A response occurred at 0.02 ms, but in the guinea-pig there was little increase in the size of contraction on further increasing the stimulus width (Figure 3a). In the rat the size appeared to reach a plateau at about 0.03 ms, and then rose again progressively as the width increased. When a stimulus width of 1 ms was reached (not shown in Figure 3a), the contractile response of the rat had reached an amplitude of 16.1% of the maximum carbachol response, whereas the guinea-pig response was still only 9.5%. Examples of the shape of the responses are shown in Figure 4, along with the effects of atropine (3×10^{-7} M), TTX (1.6×10^{-6} M) and α,β-MeATP (10^{-4} M). With pulse durations of over 0.1 ms, the increase in amplitude in the rat was clearly caused by a slow component which was superimposed on the nerve-evoked response. This was insensitive to TTX, α,β-MeATP and atropine, and probably caused by direct excitation of the smooth muscle cells. In the guinea-pig the direct muscle response was

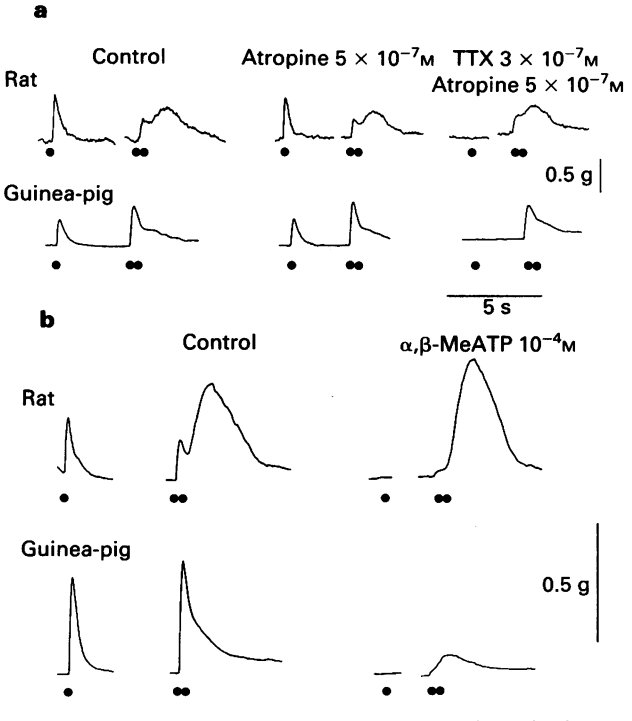

Figure 4 The contractile response of rat and guinea-pig detrusor strips to single stimuli. (a) The effects of atropine and tetrodotoxin (TTX). (b) The effects of desensitization of the P_2-purinoceptors with α,β-methylene ATP (α,β-MeATP). (●) Single shock of 0.05 ms, 50 V; (●●) single shock of 1 ms, 50 V. Note that in the rat, TTX- and α,β-MeATP-resistant response (direct muscle stimulation) appears as a separate slow component of the contraction and often reduces the size of the nerve-mediated component, whereas in the guinea-pig, it adds to the first component, and also contributes to a slower relaxation.

small and it was not clearly separated in time from the nerve evoked response. When the voltage was increased at a constant pulse width of 0.05 ms increases in response amplitude were produced that were indistinguishable in the two species (Figure 3b). Responses occurred at a threshold of about 20 V, were relatively constant between 25 and 50 V and then increased. This increase was again caused by direct stimulation of the smooth muscle cells. The response had the same characteristics as the response brought in at increasing pulse widths. It was resistant to TTX, α,β-MeATP and atropine. This direct muscle stimulation was not seen with the electrode configuration and stimulus parameters used by Bhat *et al.* (1989) or Maggi *et al.* (1985).

Effect of drugs For the investigation of the effect of drugs on nerve-mediated contractile responses, a standard stimulus of 0.05 ms and 50 V was used. Figure 4 shows that the response in both species was completely abolished by TTX (3×10^{-7} M) and by desensitization of the P_2-purinoceptors with α,β-MeATP (5×10^{-7} M). Figure 5 shows two responses of a strip of guinea-pig muscle before and after atropine (5×10^{-7} M), in which there was no obvious effect of the drug. However, because of the great variability of the responses with time, it was difficult to quantify the effects of atropine precisely and in some cases a small decrease in the size of the response was seen, although this could have been a time-dependent phenomenon. If the dose of atropine was increased to 3×10^{-6} M there was a clear decrease in the spontaneous activity of many of the tissues and there was always a decrease in the nerve-mediated response and a supression of the bi- or multiphasic activity as shown in Figure 6. It seems likely that this dose of atropine may have some non-specific inhibitory effect on the tissue.

Bi- or multiphasic responses could always be turned into monophasic responses by giving a second stimulus immediately after the first response had decayed, as shown in Figure 7. This also occurred in the presence of 5×10^{-7} M atropine. The multiphasic nature of the response became more pronounced when the spontaneous activity of the tissue was

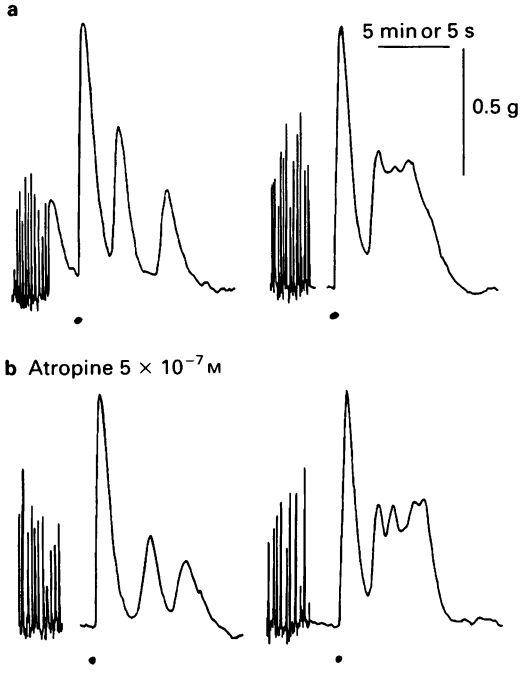

Figure 5 The effect of 5×10^{-7} M atropine on the contractile response to single stimuli (at ●, 0.05 ms, 50 V) in the guinea-pig. In this animal there was considerable spontaneous activity, and the response was multiphasic and appeared in two configurations. Atropine had little effect at this concentration. Sweep speed at faster rate for traces showing effect of electric stimulation.

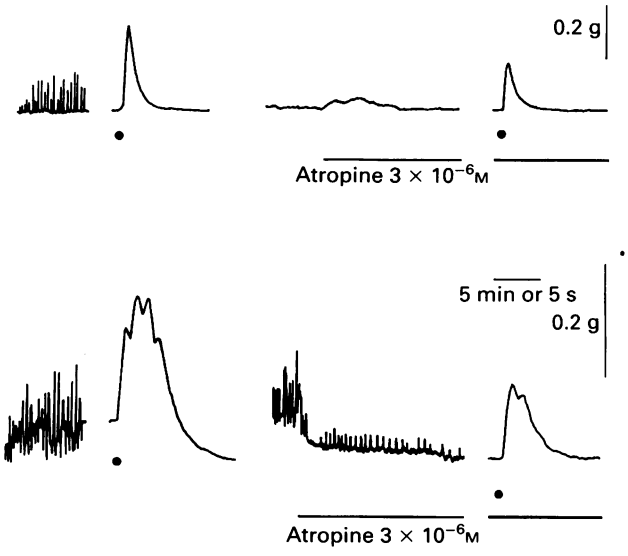

Figure 6 The effect of 3×10^{-6} M atropine on spontaneous activity and the response to single stimuli (at ●, 0.05 ms, 50 V) in guinea-pig and rat (top) detrusor. Note the reduction (bottom) of the spontaneous activity (in the guinea-pig the synchronous phasic activity was abolished), and the reduction in the amplitude of the contractile response. Sweep speed at faster rate for traces showing effect of electrical stimulation.

increased by application of TEA (1 mM), as shown in Figure 8, consistent with the results of Maggi *et al.* (1985).

Frequency-response curve

Figure 9 shows the frequency-response curve for rat detrusor strips. Five-second trains of pulses of 0.05 ms duration and 50 V were used. The maximum response of the tissues to transmural nerve stimulation was about 70% of the response to 10^{-4} M carbachol, in comparison to just over 80% in the guinea-pig (Brading, 1987). In this experiment, 10^{-6} M atropine was used for comparison with the guinea-pig data, and at this concentration there is a small, but not significant

Figure 7 The effect of a second stimulus given immediately after the response to a first stimulus (0.05 ms, 50 V) in the rat detrusor strip (stimulus marked ●). The response of two strips taken from different rats is shown. Note that the second response is a simple monophasic response.

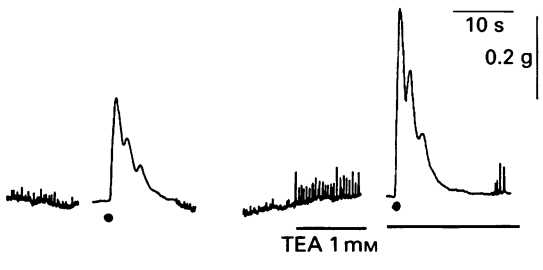

Figure 8 The effect of 1 mM tetraethylammonium (TEA) on the spontaneous activity and evoked response (single stimulus at ●, 0.05 ms, 50 V) of a strip of rat detrusor. Response in normal Krebs solution is shown on the left.

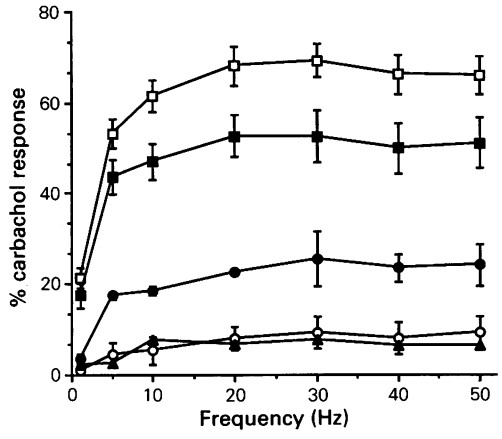

Figure 9 Frequency-response curves for rat detrusor strips: 0.05 ms, 50 V stimuli were applied at increasing frequencies for 5 s, every 10 min. Responses are mean with s.e.mean shown by vertical bars of 4–6 strips. (□) Control; (■) atropine 10^{-6} M; (●) α,β-methylene ATP (α,β-MeATP) 10^{-4} M; (○) atropine + α,β-MeATP; (▲) tetrodotoxin 1.6×10^{-6} M. Note the large atropine-resistant response, the greater effect of atropine at high frequencies of stimulation and the proportionally greater effect of desensitization of the P_2-purinoceptors with α,β-MeATP at the lower frequencies. The responses in the combined presence of atropine and α,β-MeATP are not significantly different from those in TTX.

reduction in response to stimulation at 1 Hz. As can be seen from the figure, atropine had its maximum effect on the response at frequencies above 20 Hz, and caused about 25% reduction of the contraction. The same was true in the guinea-pig (Brading, 1987). In contrast, desensitization of the P_2-purinoceptors with 10^{-4} M α,β-MeATP completely abolished the response of the tissue to a single stimulus of this duration and intensity in all tissues tested (see Figure 4), almost completely abolished the response at 1 Hz and depressed the contractile response by 73–77% at frequencies above 10 Hz. A smaller concentration of α,β-MeATP (5×10^{-6} M, not shown) depressed the response by 34% at 1 Hz, and by progressively smaller amounts until 40 Hz, when it had no effect. This again was very similar in the guinea-pig, suggesting that at this concentration not all of the purinoceptors are desensitized. The contractile responses in the combined presence of 10^{-4} M α,β-MeATP and 10^{-6} M atropine, were not significantly different from the responses in the presence of TTX, and in both cases it was noted that there was no contractile response during the stimulation period but the small contraction recorded occurred after the stimulus had ended.

Discussion

The results described in this paper confirm that in the rat, as in the guinea-pig, the contractile response of the detrusor muscle to transmural nerve stimulation consists of two components, which can be activated by different frequencies of stimulation, and that one part of the response is mediated through muscarinic receptors and the other part probably through P_2-purinoceptors. This duality of excitatory innervation has been found in all mammals studied with the exception of some old world monkeys and man (Craggs & Stephenson, 1985; Sibley, 1984), although there have been some reports (e.g. Husted *et al.*, 1983) of a non-cholinergic component in the human bladder. It has also been found, however, that the responses to low frequencies of stimulation are very resistant to atropine (see Brading, 1987), the cholinergic component of contraction appearing as the frequency of stimulation is increased. The suggestion has recently been made that in the rat, the contractile response to a single transmural nerve stimulus also has two components, the second of which may be mediated by activation of muscarinic receptors (Maggi *et al.*, 1985; Bhat *et al.*, 1989). Bhat and his colleagues have shown that the second component is somewhat more susceptible to blockade with Ca-channel blocking drugs, suggesting that Ca entry through voltage-sensitive Ca channels may be involved. Interestingly however, they found that the response to bath-applied acetylcholine was less sensitive to Ca antagonists.

It has recently been demonstrated that the response to muscarinic receptor activation in the guinea-pig detrusor does not involve significant depolarization of the cell membrane, whereas the response to ATP and to single nerve stimuli both involve marked depolarization of the membrane (Fujii, 1988). If the situation is similar in the rat, then it would be expected that the second component of the contraction, if it were indeed cholinergic, would be more resistant to Ca-antagonists than the early non-cholinergic response. We have therefore compared the contractile responses of the rat with those of the guinea-pig detrusor to transmural stimulation.

The strength-duration curves we have recorded are similar to those obtained in the rabbit and the pig by Sibley (1984). The curves are shifted to the right in the presence of TTX, suggesting that the contractile response at threshold voltages is caused by an action potential in the transmural nerves releasing an excitatory transmitter. The predominant transmitter is clearly not acetylcholine, since atropine has no effect on the strength-duration curves in either the rat or the guinea-pig. The transmitter seems to work through P_2-purinoceptors, since desensitization of these with α,β-MeATP also shifts the curves to the right, eliminating the response of the tissue to nerve-mediated stimuli. This alone suggests that acetylcholine is not playing a significant role in contractile response to single stimuli, since, in the guinea-pig at least, α,β-MeATP is specific in its actions, and does not affect the tissue response to acetylcholine (Fujii, 1988).

The responses to transmural stimulation which persist in the presence of TTX and α,β-MeATP are presumably due to direct activation of the smooth muscle cells, and it is interesting to note that with sufficient voltage, the cells can be activated by very short pulses. In the rat, direct muscle stimulation with a single pulse can produce a contractile response considerably larger than the maximum nerve-mediated response, whereas in the guinea-pig there is only a relatively small increment. The fact that the strength-duration curve in human tissues is not affected by TTX, is consistent with the lack of a non-cholinergic excitatory transmitter in man.

We found it difficult to study the effect of drugs on the shape of the contractile response to a single nerve stimulus, because of its variability.

We would agree with Maggi *et al.* (1985) and Bhat *et al.* (1989) that atropine at 3×10^{-6} M suppresses the response and that this effect is more marked on the second component where present, but this is a very high concentration of atropine and probably lowers the excitability of the tissue, as shown by a reduction in the spontaneous activity. At the more selective concentration of 5×10^{-7} M (in which the response to 10^{-4} M carbachol was virtually abolished) it was no longer clear that the second response was selectively suppressed. In some instances the whole response was somewhat reduced by this dose of atropine, although this might have been a time-dependent depression and in some instances the response was little affected or even bigger. These results are not sufficiently clear to allow us to rule out a minor contribution of acetylcholine to the later components of the response. However, since it is clear from the frequency-response curves that the cholinergic component becomes more significant with repetitive stimulation, we looked at the effect of giving two pulses close together. In this instance, the results are extremely clear cut. Giving the second stimulus immediately after the first response had decayed to baseline, invariably results in the second response being monophasic, even if the response to the first stimulus was multiphasic. This does not support a role

for acetylcholine in the later contractions. It is probable that the smooth muscle cell membranes are less excitable for a short while after the stimulus.

Our experiments suggest that the shape of the response to a single nerve stimulus is strongly dependent on the excitability of the tissue. It is closely correlated with the degree of spontaneous activity of the tissue, and the shape becomes simpler as the spontaneous activity decreases. In the experiments carried out by Maggi *et al.* (1985), the tissue was continuously stimulated at 0.01 Hz, which seems to suppress spontaneous activity, and in the paper by Bhat *et al.* (1989) no reference is made to the spontaneous activity. In agreement with Maggi *et al.* (1985) we find that the size of the single response and the amplitude of the secondary components are increased in the presence of tetraethylammonium, but we also show that this drug increases the spontaneous activity of the tissue.

We feel that the most likely explanation for the later components of the contractile response to single nerve-mediated stimuli, may be repetitive firing of action potentials triggered by an excitatory junction potential resulting from activation of P_2 purinoceptors and that the number of action potentials and the size of the contractile response to them may depend to a large extent on the excitability of the tissue. It is possible that simultaneous release of acetylcholine does modify the excitability of the tissue, either at the level of the cell membrane, or by release of intracellular calcium bringing the cells nearer to their contractile threshold. In the rabbit, the response to a single nerve stimulus consists of a fast atropine-resistant excitatory junction potential with superimposed spikes, followed by a slower depolarization which is blocked by atropine (Creed *et al.*, 1983; Fujii, 1988). However, this secondary depolarization is not evident in the guinea-pig (Fujii, 1988) under normal conditions, and similar experiments have not been carried out on rat bladder. In the rabbit (Sibley, 1984), the cholinergic component of the contractile response at high frequencies is larger than in the rat and guinea-pig (up to 40% as compared with 25% in the rat and guinea-pig).

If the role of acetylcholine in the response to a single nerve stimulus is to modify the excitability of the cells, then this could explain the difference seen by Bhat and his colleagues (1989) in the susceptibility of the response to bath-applied acetylcholine and the second component of the response to single stimuli to Ca-antagonist drugs, and the lowering of extracellular calcium.

In conclusion, the excitatory innervation of the rat and guinea-pig detrusor is very similar. The predominant innervation is non-cholinergic, and the cholinergic component of the contractile response is most marked with repetitive stimulation at frequencies of 10 Hz and above, when it can generate 25% of the contraction. The non-cholinergic transmitter is dominant in the response to single nerve stimuli, the size being unaffected by blockade of muscarinic receptors, but blocked by desensitization of the P_2-purinoceptors with α,β-MeATP. The shape of the response is influenced by the excitability of the tissue, and this may be modified by the cholinergic component.

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