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Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation

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1 The electrical and mechanical responses to transmural stimulation of intrinsic nerves have been recorded from smooth muscle strips dissected from the dome of the guinea-pig bladder, by use of intracellular microelectrodes, and conventional tension recording techniques.

2 Stimulation of intrinsic nerves evoked action potentials in all cells studied. Hyperpolarization of the cells by extracellular current injection revealed subthreshold excitatory junction potentials (e.j.ps) in about a quarter of the cells studied.

3 Action potentials could still be evoked in the presence of atropine and neostigmine, but were abolished after desensitization of the cells to α,β-methylene ATP, a stable analogue of ATP.

4 In the presence of neostigmine, the evoked action potential was followed by a slow depolarization of the membrane. The mechanical response increased in amplitude and duration.

5 The contractile response to transmural nerve stimulation was reduced but not abolished in the presence of either atropine or desensitizing doses of α,β-methylene ATP. Atropine was more effective at high frequencies of stimulation ($\geqslant 30$ Hz), and α,β-methylene ATP at low frequencies ($\leqslant 15$ Hz). In combination the drugs abolished the response.

6 The results suggest that the mechanical response to excitatory nerve stimulation is biphasic. The early transient reponse is elicited by e.j.ps and evoked spikes, is resistant to atropine, but sensitive to desensitization of purinoceptors. The late response is mediated through muscarinic receptors, involves little membrane depolarization, and is unaffected by desensitization of purinoceptors. These responses are analogous to the responses seen in rabbit bladder, and in the sympathetically innervated rat tail artery and guinea-pig vas deferens.

Introduction

The atropine resistance of the contractile response to nerve stimulation of the mammalian urinary bladder was first recognized by Langley & Anderson (1885). The role of acetylcholine in excitatory transmission in the bladder was studied later in more detail by Henderson & Roepke (1934), who again noted that atropine does not completely abolish the response to nerve stimulation. Recent evidence has suggested that adenosine 5'-triphosphate (ATP) may be the neurotransmitter responsible for atropine resistant responses. ATP is released from guinea-pig bladder upon nerve stimulation, and mimics the non-cholinergic, non-adrenergic contractile response when applied locally to the muscle (Burnstock *et al.*, 1972; 1978). ATP has also been shown to be stored in guinea-pig bladder, although it is not known whether it is co-located with acetylcholine in cholinergic nerves, or is in separate purinergic nerves (Burnstock, 1983).

Creed *et al.* (1983) have recorded the electrical responses of rabbit bladder smooth muscle to single stimuli of the intrinsic nerves, and have observed an initial electrical response consisting of an excitatory junction potential (e.j.p.) giving rise to a spike, and a delayed more prolonged depolarization of 250–300 ms latency and 2 s duration. The initial response was unaffected by atropine or neostigmine, whilst the delayed response was abolished by atropine, and enhanced by neostigmine. Subsequently, Hoyle & Burnstock (1985) showed that the e.j.p. of rabbit bladder, recorded by use of sucrose-gap apparatus, was abolished after desensitization with α,β-methylene ATP. Also, Callahan & Creed (1986), using a similar technique, have recorded e.j.ps giving rise to

action potentials in guinea-pig bladder that are insensitive to phentolamine and atropine.

These responses in mammalian bladder are reminiscent of the situation in several adrenergically innervated tissues, such as the guinea-pig vas deferens and rat tail artery. In these tissues similar biphasic responses to nerve stimulation have been recorded (Sneddon & Westfall, 1983; Sneddon & Burnstock, 1984) which can be differentially affected by α-adrenoceptor antagonists and α,β-methylene ATP, leading to the suggestion that ATP and noradrenaline acts as co-transmitters in these two systems (Sneddon & Burnstock, 1985).

The evidence suggests that in both the adrenergic and cholinergic systems discussed above, the biphasic response to nerve stimulation may be analogous. In each system there is an early tightly coupled electrical response in the form of an e.j.p. which may give rise to an action potential and can be abolished by purinoceptor desensitization, and a more latent response which is mediated by the classical neurotransmitter.

The experiments presented here were undertaken to characterize the electrical and mechanical responses of the urinary bladder of the guinea-pig to nerve stimulation and to investigate the possible contribution of a purinergic transmitter to the observed responses. Previous studies of the mechanical responses of guinea-pig bladder muscle have suggested that co-transmission in this structure may involve acetylcholine and a non-cholinergic non-adrenergic, possibly purinergic, transmitter (Mackenzie *et al.*, 1982; Theobald, 1982; Moss & Burnstock, 1985).

Methods

Male albino guinea-pigs weighing 300–600 g were stunned and bled. Strips of urinary bladder muscle were removed from the freely moveable intraperitoneal portion.

For mechanical recordings, strips measuring about $10 \times 1 \times 1$ mm were mounted in 0.2 ml organ baths containing platinum ring electrodes (Brading & Sibley, 1983). Tension was measured isometrically by use of Pioden transducers, with their output recorded on a six channel Watanabe pen recorder. Field stimulation of the intrinsic nerves was achieved with rectangular pulses from a Grass S48 stimulator (50 V, 50 μs, 1 s trains, variable frequency). After being mounted, tissues were allowed to equilibrate for one hour before exposure to a supramaximal concentration of carbachol (10 μM for 10 s). Subsequent responses were expressed as a percentage of this maximum response (mean $\pm$ s.e.mean).

Intracellular microelectrodes were pulled with resistances of 30–40 MΩ, filled with 3 M KCl. Tissue strips measuring $8 \times 5 \times 1$ mm were placed in a chamber equipped with an insulated partition for extracellular current application (Abe & Tomita, 1968), and an additional pair of silver ring electrodes round the tissue in the main bath for intrinsic nerve stimulation. The chamber was perfused with modified Krebs solution at 32–34°C, and responses were amplified and recorded on magnetic tape for subsequent reproduction on a digital oscilloscope and pen recorder.

A modified Krebs solution was used containing (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $CaCl_2$ 2.5, glucose 11, equilibrated with 97% O_2/3% CO_2, pH 7.4 at 36°C. Sucrose (15 g 100 ml^{-1}) was added to solutions used for all microelectrode experiments to dissociate action potentials from contraction and thus prevent electrode dislodgement. This treatment has little effect on the electrical properties of the membrane (Tomita, 1966; Creed, 1971).

All drugs were obtained from Sigma and dissolved in the perfusing solution. The concentrations stated are the final bath values. Student's t test was used to calculate the P values given in the paper; P values of less than 0.05 were considered to be significant.

Results

Membrane responses to electrical stimulation

The resting membrane potential of a sample of cells impaled in the course of these experiments was -38.5 ± 2.3 mV ($n = 25$). Most cells showed spontaneous spike activity. Stimulation of the intrinsic nerves was attempted with brief electrical pulses. With increasing duration (beyond 100 μs) an intensity of threshold could be found in each preparation at which a single pulse would evoke an action potential that could be blocked by tetrodotoxin (TTX) (0.2 μg ml^{-1}), and was presumably evoked by nerve stimulation. Brief trains of stimuli (2–60 Hz) were more effective at initiating action potentials, but with increasing duration and strength of stimulation TTX-resistant action potentials were evoked, presumably due to direct muscle activation.

Current pulses of 1000–1200 ms duration were applied by use of partition electrodes to hyperpolarize cells close to the insulated partition. Suprathreshold stimulation of the intrinsic nerves through the ring electrodes was applied during conditioning hyperpolarization. The evoked action potentials could be suppressed with increasing hyperpolarization. In about 1 in 4 or 5 of the cells penetrated, an excitatory junction potential (e.j.p.) could be

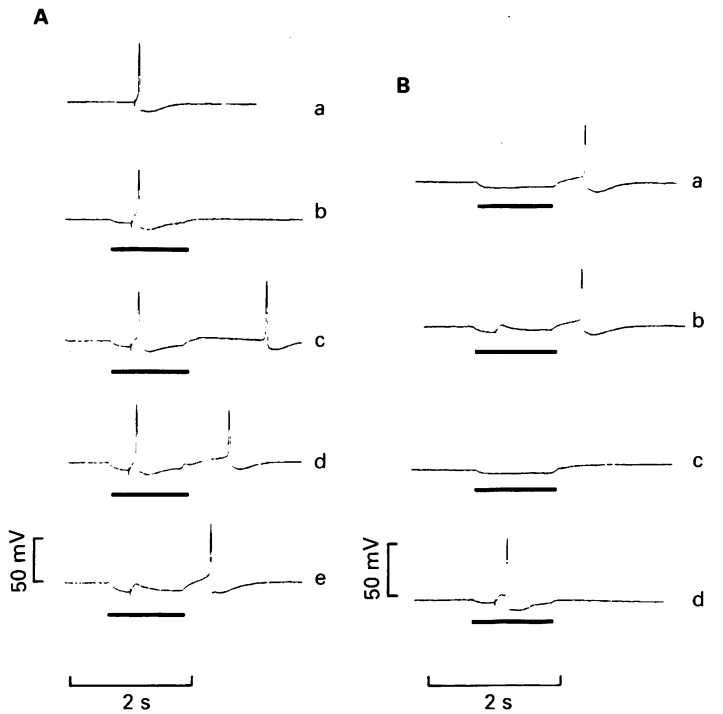

Figure 1 Microelectrode recordings of the effect of conditioning hyperpolarization on the membrane response to stimulation of intrinsic nerves with brief electrical stimuli. (A), (a) Control response with no conditioning hyperpolarization; (b–e) conditioning hyperpolarizing current of increasing strength from 0.2–0.8 mA was applied through the partition electrodes for 1.2 s beginning 300 ms before the nerves were stimulated (ring electrodes, 100 μs 100 V). In (e) the hyperpolarization is sufficient to prevent the e.j.p. reaching threshold for spike initiation. Note the appearance of anode break excitation with the larger hyperpolarizing currents. Recordings from the same cell. Resting membrane potential on initial impalement −38 mV. (B) In another cell; (a and c) conditioning hyperpolarization alone (1.2 s, 0.8 mA); (b) nerve stimulation (100 μs, 80 V) 300 ms after onset of the conditioning hyperpolarization results in an e.j.p.; (d) increasing the strength of the nerve stimulation to 100 V now results in an e.j.p. giving rise to an action potential. Resting membrane potential on impalement −38 mV.

recorded after the action potential had been suppressed. Figure 1A shows the effect of increasing the conditioning hyperpolarization on the evoked response of a single cell. In this cell a clear e.j.p. was visible after suppression of the action potential. In another cell (Figure 1B), after suppression of the action potential with conditioning hyperpolarization, increasing the strength of the nerve stimulation caused a return of the evoked action potential.

E.j.ps of a duration between 400–600 ms, and size from 8–12 mV in amplitude, could be recorded during conditioning hyperpolarization, but since there was considerable cell-to-cell variation in the absolute level of membrane potential at which the action potential was abolished, the driving force for the excitatory junctional current will be different in each case.

In a $0Ca^{2+}$ solution the e.j.ps and evoked action potentials were abolished before the spontaneous action potentials were affected (see Figure 2), consistent with the suggestion that the evoked responses are e.j.ps and not subthreshold direct membrane responses to the stimulus. Because of the relative infrequency and unpredictability of recording evoked e.j.ps, in the remainder of the experiments action potentials evoked by stimulus parameters chosen such that the responses were abolished by TTX (nerve-evoked action potentials), were recorded to assess the effects of drugs on nerve-mediated activity.

The effect of drugs on membrane activity

Atropine (1 μM), at a concentration sufficient to block the response to exogenous administered carbachol (10 μM), did not prevent nerve stimulation from evoking action potentials (see Figure 3). Application of neostigmine (1 μM) had little effect on the configu-

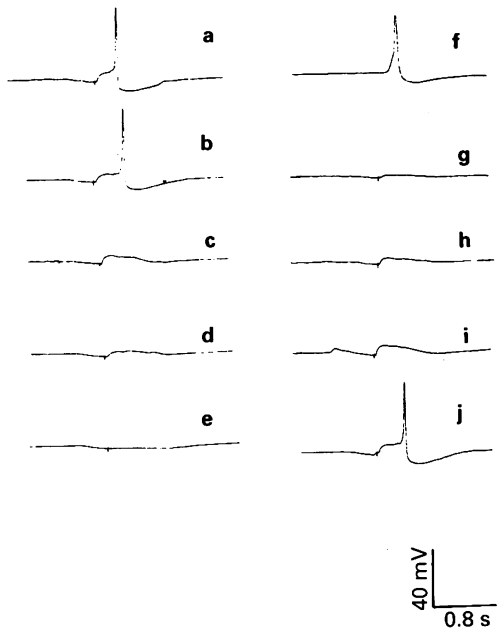

Figure 2 Effect of Ca^{2+} removal on the membrane responses to transmural nerve stimulation (100 μs, 100 V) applied 300 ms after the beginning of a conditioning hyperpolarizing current (1.2 s, 0.4 mA). Resting membrane potential on initial impalement −38 mV. (a) In normal Krebs solution an e.j.p. was elicited which gave rise to an action potential. (b) 90 s, (c) 2 min, (d) 2.5 min, (e) 3 min after changing to 0 Ca Krebs (6 mM Mg). Note progressive reduction in size of the e.j.p. and loss of the spike. (f) Spontaneous spike occurring as solution is changed to readmit normal Krebs solution. (g) 90 s, (h) 2 min, (i) 2.5 min, (j) 3 min after readmission of normal Krebs solution. Note the appearance of an e.j.p. and spike. Recordings from a single cell.

ration of the evoked spikes, although there was sometimes a small increase in spike amplitude (see Figure 3). Following exposure to 30 μM neostigmine, a slow depolarization of 5–10 mV and 15–20 s duration appeared after each action potential (see Figure 4). Superimposed on these were small spontaneous transient depolarizations.

To test the possibility that ATP might be responsible for the atropine-resistant response to nerve stimulation, tissues were desensitized to the non-hydrolyzable analogue of ATP, α,β-methylene ATP, by exposing them to progressively higher concentrations beginning with 1 μM (5 min), then 5 μM (5 min) and finally 30 μM (continuous). When the substance was first applied to the tissue, there was a transient membrane depolarization and an increased frequency of action potentials, which made it difficult to hold an impalement with microelectrode. Excitatory responses, as judged by the contractile behaviour, diminished with subsequent doses, and there was no effect on application of 30 μM. To study the effect of the drug on nerve-evoked action potentials, microelectrode recordings were made from cells before application of the drug, and further penetrations were then only attempted after desensitization was complete. Figure 5 illustrates the results of one such experiment in which tissues were stimulated by trains of very brief electrical stimuli (50 μs) at different frequencies before and after desensitization of the tissues to α,β-methylene ATP. Before treatment with the drug, action potentials were evoked with a latency that decreased with increasing stimuli to the intrinsic nerves. After treatment, actions potentials could not be evoked by any frequency, although spontaneous action potentials were still seen.

The effect of drugs on contractile activity

The effect of atropine (1 μM) on the contractile response to nerve stimulation was frequency-dependent (see Figure 6). There was little effect at low frequencies, but at higher frequencies atropine reduced the size of the contraction by a maximum of about 30%. Neostigmine enhanced the contractile responses at all frequencies of nerve stimulation as seen in Figure 7. Individual responses are illustrated in Figure 8, showing that not only is there an increase in the maximal size of the response, but it becomes greatly prolonged, and contains oscillatory components (also seen on application of acetylcholine, (Mostwin, 1985)). The fact that the effects of neostigmine are antagonized by atropine (see Figure 7 and 8), suggests that the effect is predominantly through muscarinic receptor activation, and thus postganglionic. After desensitization of the tissue to α,β-methylene ATP, the nerve-evoked contractile response was reduced at all frequencies, but the percentage reduction was greatest at low frequencies of stimulation (see Figure 6). A combination of desensitization to α,β-methylene ATP and atropine (1 μM) abolished the nerve-evoked response at all frequencies. The residual small contractile response evoked at high frequencies was also resistant to TTX (0.2 $\mu g\,ml^{-1}$), and thus presumably a response to direct muscle stimulation.

Discussion

These findings support and extend earlier observations that neuromuscular transmission in the smooth muscle of the guinea-pig bladder may involve both a muscarinic and a purinergic component.

The e.j.ps recorded are similar to e.j.ps recorded by microelectrode from other smooth muscles. In the guinea-pig vas deferens, e.j.ps of 8–10 mV amplitude

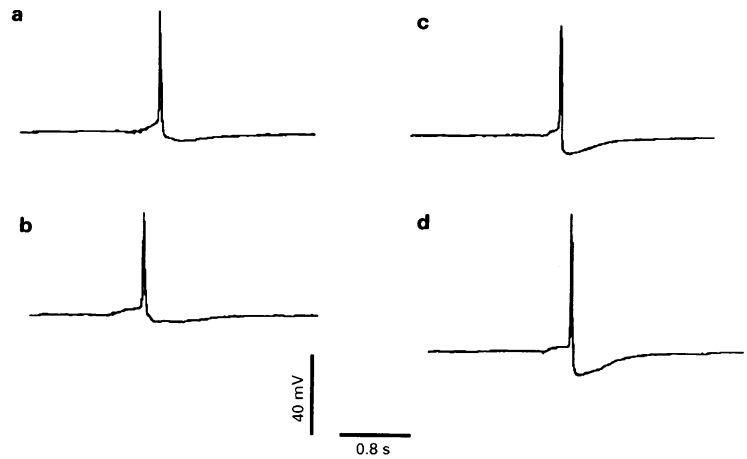

Figure 3 Effect of atropine (1 μM) and neostigmine (1 μM) on the electrical response to nerve stimulation. (a and c) Controls in normal Krebs solution; (b) 30 min after application of atropine; (d) 30 min after application of neostigmine. (a and b) From a single cell, (c and d) from another cell. There is little effect of atropine on the membrane response, but the action potential in the presence of neostigmine was increased in amplitude. Initial membrane potential of both cells −42 mV.

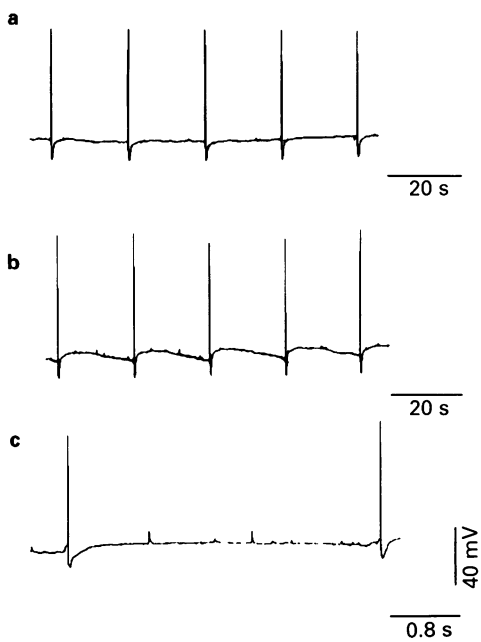

Figure 4 Effect of neostigmine on the membrane response to repetitive nerve stimulation (100 μs, 100 V every 20 s). Initial membrane potential −42 mV. The records are a continuation of Figure 3d. (a) After 40 min in neostigmine (1 μM), (b) 10 min after increasing the neostigmine concentration to 30 μM the action potential is followed by a slow depolarization of 5–10 mV, with brief spontaneous transient membrane depolarization superimposed. (c) Part of the recording in (b) with expanded time scale. All recordings from the same cell.

and 600 ms duration (Sneddon & Westfall, 1983) or 15 mV amplitude and 500–600 ms duration (Sneddon & Burnstock, 1984) have been recorded, and in the rat tail artery Sneddon & Burnstock (1985) have recorded e.j.ps of 8–10 mV amplitude. In the guinea-pig bladder, isolated e.j.ps were not found unless a positive conditioning current was used, which suggests that the e.j.ps normally cross the threshold for the initiation of action potentials. The proposal that the resting membrane potential of guinea-pig bladder is close to the threshold action potential excitation has been made previously (Brading *et al.*, 1986). The finding that discrete e.j.ps could not be recorded from every cell penetrated suggests that close connections with nerve varicosities may not occur for every smooth muscle cell. The density of innervation in bladder smooth muscle has been assessed by Gosling *et al.* (1982) who have reached a similar conclusion from electron microscopy.

The observations presented here are analogous to previous findings in rat tail artery and guinea-pig vas deferens, two tissues innervated by sympathetic adrenergic nerves, in which electrophysiological evidence for co-transmission of noradrenaline and ATP has been found. In each tissue nerve, stimulation results in an early rapid transient component which is abolished by arylazidoaminopropionyl ATP ($ANAPP_3$) or desensitization to α,β-methylene ATP, and a prolonged (30 s in rat tail artery) latent depolarization which is sensitive to phentolamine (Sneddon & Westfall, 1983; Sneddon & Burnstock, 1984; 1985). In the guinea-pig bladder, the co-transmission involves a cholinergic, parasympathetic innervation. There is no evidence for a direct sympa-

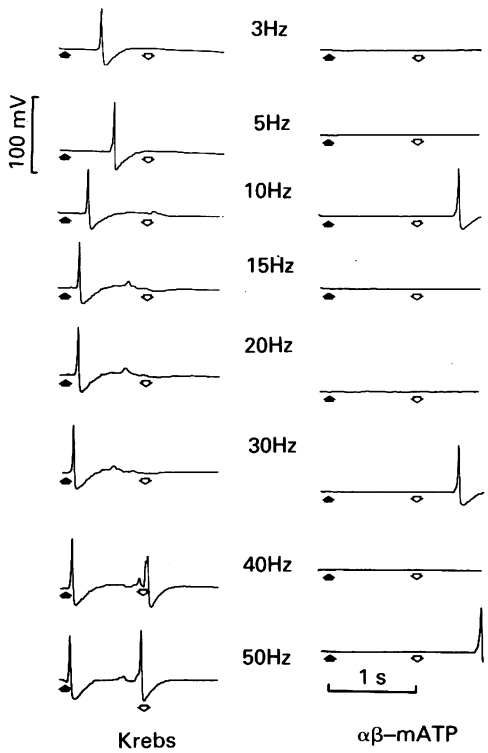

Figure 5 Effect of desensitization to α,β-methylene ATP (α,β-mATP) on the membrane response to nerve stimulation elicited by 1 s trains of increasing frequency (50 μs, 50 V, indicated by the arrows on the baseline of each tracing). Response in Krebs solution on the left, and after desensitization to α, β-methylene ATP on the right. The isolated action potentials among the tracings on the right are spontaneous. Records on the right from a single cell, and on the left from another cell in the same preparation. Initial resting membrane potential of both cells −38 mV.

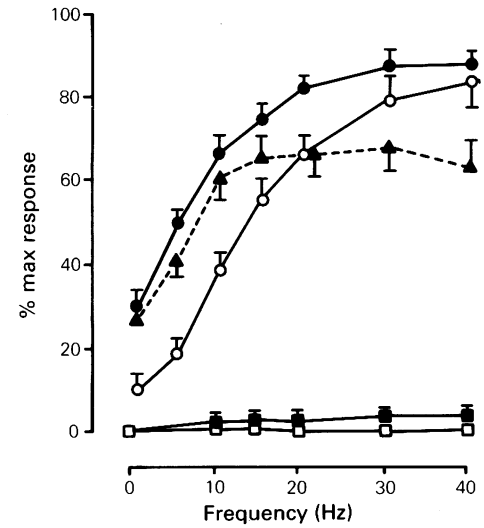

Figure 6 Effect of atropine and desensitization to α,β-methylene ATP on the contractile response of bladder strips to stimulation of the intrinsic nerves with trains of stimuli (50 μs, 50 V, 1 s train duration), stimulation frequency on abscissa scale. (●) Control responses in Krebs solution, (▲) responses in atropine (1 μM), (○) responses after desensitization of the preparation to α,β-methylene ATP (30 μM). The differences between the control responses and those in the presence of atropine are significant at 30 and 40 Hz, and between the control responses and after desensitization with α,β-methylene ATP and atropine (■). There is no significant difference between these responses and those in TTX (0.2 μg ms^{-1}) (□). Each point represents the mean (n = 5) and vertical lines show s.e.mean.

thetic innervation of the dome of the guinea-pig bladder. The smooth muscle is not responsive to α- or β-adrenoceptor agonists (Edvarsen & Setekleiv, 1968; Dumsday, 1971), and the e.j.p. and action potential recorded by the sucrose-gap method are unaffected by phentolamine (Callahan & Creed, 1986).

The effect of atropine on the contractile response seen in these experiments was greater at stimulation frequencies ⩾ 30 Hz. The effect of desensitization to α,β-methylene ATP was greater at stimulation frequencies < 15 Hz. This is in general agreement with previous studies of guinea-pig bladder (De Sy, 1971; Moss & Burnstock, 1985), rabbit and pig bladder (Sibley, 1984) in which atropine resistance has been observed at stimulation frequencies < 10 Hz. Creed et al. (1983) have also noted that the late atropine-sensitive component of the electrical response of rabbit bladder to nerve stimulation increased in amplitude and duration with higher frequencies of stimulation. Kasakov & Burnstock (1983) showed that α,β-methylene ATP abolished the contractile response at stimulation frequencies > 2–4 Hz. Moss & Burnstock (1985) demonstrated the effects of atropine and α,β-methylene ATP on the contractile responses of guinea-pig, marmoset and ferret urinary bladder: desensitization to α,β-methylene ATP produced the greatest inhibition at frequencies of < 10 Hz, and with the simultaneous addition of atropine the response to field stimulation was abolished at all frequencies between 2–40 Hz. All these results, and the present data are consistent with a model in which single nerve stimuli evoke e.j.ps through release of ATP, which initiate action potentials and contraction of the detrusor. At higher frequencies, acetylcholine release triggers contraction through a mechanism which may be less dependent on depolarization of the cell membrane. The latent muscarinic depolarization seen clearly in the rabbit

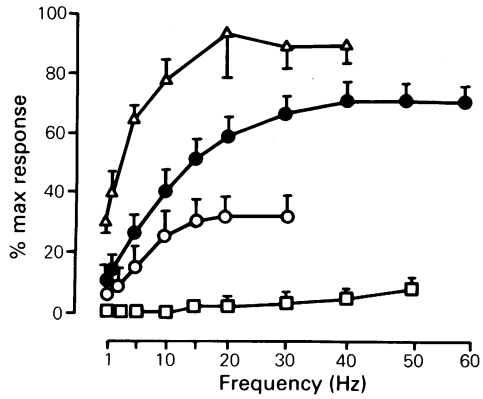

Figure 7 Effect of neostigmine in the presence and absence of atropine and α,β-methylene ATP on the contractile responses of strips of bladder to intrinsic nerve stimulation by trains of stimuli (50 μs, 50 V, 1 s train duration, stimulation frequency on abscissa scale). (●) Control responses in Krebs solution. (△) Responses in neostigmine (1 μM). (○) Responses in the presence of neostigmine and atropine. (□) Responses in the presence of neostigmine and atropine, after desensitization of the tissue to α,β-methylene ATP (30 μM).

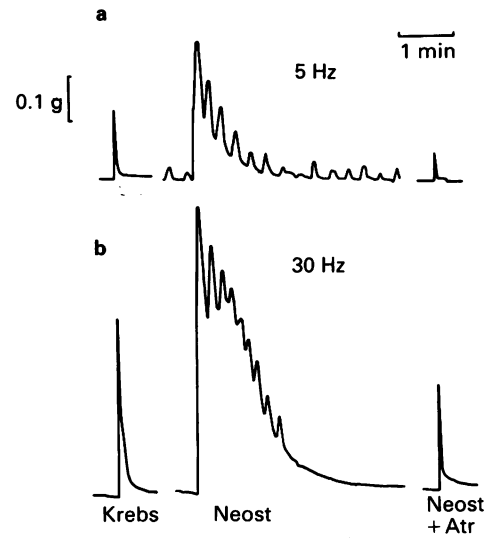

Figure 8 Effect of neostigmine on the mechanical responses of two strips of bladder to stimulation of the intrinsic nerves with 1 s trains (50 μs, 50 V) at 5 Hz (a) and 30 Hz (b). Left column shows responses after 20 min in neostigmine (Neost, 1 μM). Right column shows responses 20 min after exposure to atropine (Atr, 1 μM) in the continued presence of neostigmine.

is only seen in the presence of cholinesterase inhibitors in the guinea-pig, although there is a significant muscarinic component to contraction in their absence.

During preparation of this manuscript, further work carried out on this problem and extending the above results has been published (Fujii, 1988).

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