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RAT ISOLATED PHRENIC NERVE-DIAPHRAGM PREPARATION FOR PHARMACOLOGICAL STUDY OF MUSCLE SPINDLE AFFERENT ACTIVITY: EFFECT OF OXOTREMORINE

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1 Muscle spindle afferent discharges exhibiting an approximately linear length-frequency relation could be recorded from the phrenic nerve in the isolated phrenic nerve-diaphragm preparation of the rat.

2 Muscle spindle afferent discharges could be identified by their characteristic 'spindle pause' during muscle contraction and by their response to succinylcholine.

3 Cholinergic influence on spontaneous and stretch-induced afferent discharges was indicated by the augmentation produced by physostigmine and acetylcholine. (+)-Tubocurarine, but not atropine, prevented this augmentation indicating the presence of curariform cholinoceptors in muscle spindles.

4 Acetylcholine did not appear to be involved in the genesis of spindle afferent discharges as incubation with hemicholinium-3 and (+)-tubocurarine failed to affect the rate of spontaneous and stretch-induced spindle discharges.

5 Oxotremorine markedly increased the rate of spontaneous and stretch-induced spindle afferent discharges and this effect was prevented in the presence of hemicholinium-3 and (+)-tubocurarine.

6 These results with oxotremorine are of interest in connection with the observation that muscle spindle afferents are hyperactive in Parkinsonian patients.

Introduction

There has been a considerable number of investigations on the influence of drugs on fusimotor functions and spindle afferent activity (for references, see Smith, 1963; Paintal, 1964; Matthews, 1964; 1972). However, as far as mammalian muscle spindles are concerned the assessment of pharmacological experiments is hampered by the lack of a suitable isolated preparation which would allow an unequivocal differentiation between direct and systemic drug effects. Results obtained from frog muscle spindles *in vitro* can hardly compensate for this deficit since amphibian spindles are organised in a different manner and, unlike those of mammals, do not possess a separate fusimotor control (for references, see Barker, 1974). It was therefore felt that there was scope for development of a simple *in vitro* preparation for study of pharmacological aspects of proprioceptive activity in mammalian striated muscle. To this end we have extended the work of Barstad, Kristoffersen, Lilleheil & Stalland (1965) on muscle spindle afferents in the rat phrenic nerve-diaphragm preparation with the particular purpose of testing the validity of this preparation for drug effect evaluation.

Since oxotremorine, which is unique in producing Parkinson-like motor disturbance in experimental animals, has recently been shown to increase the rate of Ia afferent discharge in decerebrate cats with intact stretch reflex loop (Fackler, Ross, Cleveland & Haase, 1977), our present study was primarily focused on the question as to how much of this effect of oxotremorine is of peripheral origin.

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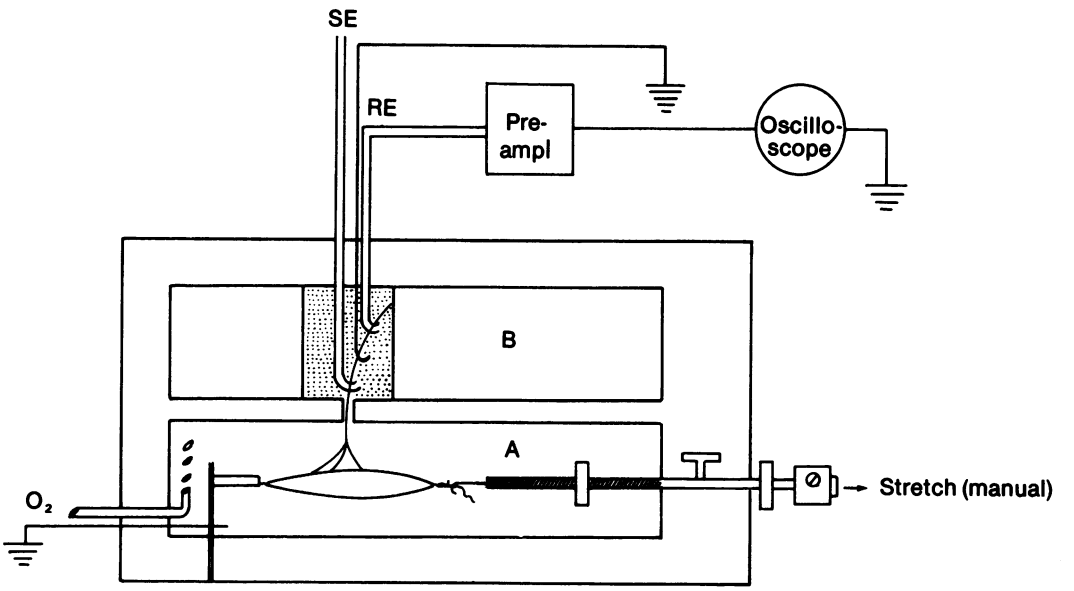

Figure 1 Diagram of the experimental set-up showing the bath with position of recording (RE) and stimulating (SE) electrodes (for details, see Methods).

Methods

The rat diaphragm together with the left phrenic nerve was dissected out according to the method of Bülbring (1946), except that only 1 to 1.5 cm of the diaphragmatic muscle was taken out around the point of entry of the phrenic nerve. Care was taken to exclude the tendinous portion and to incise the muscle along the direction of its fibres. The preparation was then transferred to a two chamber bath with an arrangement for application of manual stretch (0.5 to 4.0 mm). One end of the muscle was rigidly fixed and the other end was attached to a metal hook for applying stretch. A schematic diagram of the bath showing the position of the electrodes and muscle is given in Figure 1.

The muscle chamber (A) contained Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 24.8, dextrose 11.1, KH_2PO_4 1.2 and $MgSO_4$ 1.2, bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 31 to 32°C. The whole phrenic nerve was passed through a groove sealed with vaseline to a second chamber (B) containing liquid paraffin and was placed on two bipolar electrodes (one for stimulation and the other for recording) as shown in Figure 1. The signals from the recording electrode were led through a suitable preamplifier, displayed on an oscilloscope, and photographed. From these photographs (compare Figure 4) the static activity of the spindle afferents was determined at a series of different lengths of the diaphragm. The preparation was washed every 5 min either with normal Krebs solution during control observations or with Krebs solution containing drug during experiments.

Drugs

The following drugs were used: acetylcholine chloride (E. Merck), atropine sulphate (E. Merck), physostigmine (eserine) sulphate (E. Merck), hemicholinium-3 (Aldrich), oxotremorine sesquifumarate (Aldrich), succinylcholine chloride (Glaxo) and (+)-tubocurarine chloride (Sigma).

Results

A total of 72 experiments were performed. The preparations were allowed to acclimatize for at least 1 h after the phrenic nerve had been placed on the recording electrodes. During this period the afferent discharge rate varied considerably but then stabilized and remained quite constant up to 5 h. Preparations in which no stable activity could be obtained during the control period were discarded.

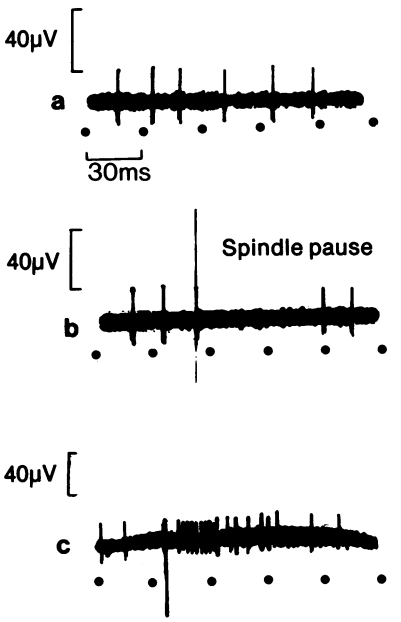

Figure 2 Identification of muscle spindle afferent discharge showing (a) constant discharge rate during a 1 mm stretch of the diaphragm; (b) 'spindle pause' during contraction of the diaphragm due to a single threshold shock to the phrenic nerve and (c) burst response following stimulation of the phrenic nerve at a high stimulus intensity (4 times the threshold for contraction). Large biphasic deflection = stimulus artefact.

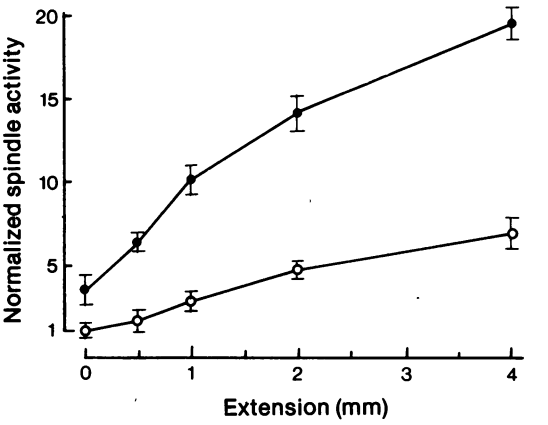

Figure 3 The dependence of static muscle spindle afferent activity on extension, determined on stretching the diaphragm to a series of different lengths. (○): Control; (●): activity after incubation with oxotremorine (0.02 mM). All values of spindle afferent activity are normalized to the mean rate of discharge at 0 mm extension during the control period. Points are mean of six experiments; vertical lines show s.e. means.

Identification of muscle spindle discharges

Discharges from the phrenic nerve were classified as originating from muscle spindle afferents, if the ongoing activity at a constant length of the muscle ceased during an isometric contraction elicited by a single electric shock to the phrenic nerve ('spindle pause'). The method of identification is shown in Figure 2a and b.

If the stimulus intensity was raised more than 3 to 4 times the threshold necessary for contraction, repetitive activity was often noticed after a single shock so that the 'spindle pause' was filled (Figure 2c). These observations are in agreement with those of Barstad *et al.* (1965). Although gamma excitation might contribute to these repetitive discharges, some interference by antidromic action potentials (Randić & Straughan, 1963; 1964) could not be excluded since the recordings were from the nerve trunk containing both afferent and efferent fibres.

Further identification of spindle afferent discharges was possible by their response to succinylcholine (Corda, Von Euler & Lennerstrand, 1965). Succinylcholine in the dose range of 0.005 to 0.02 mM augmented the rate of spontaneous and stretch-induced afferent discharges (Figure 4).

In the present study no attempt was made to differentiate afferent discharges of primary(Ia) and secondary(II) muscle spindle endings. Afferent discharges, which according to the criteria of Matthews (1972) could be classified as originating from Golgi tendon organs, were encountered in only 5 experiments. They exhibited a higher threshold to stretch and an increased activity during muscle contraction. When subjected to the succinylcholine test these afferents were silenced which is in accordance with the observations of Corda *et al.* (1965) in cat diaphragm *in situ*.

Response to muscle stretch

The rate of muscle spindle afferent discharge increased as a function of increasing amount of stretch. In the present experiments the diaphragm was stretched manually in the range of 0 to 4 mm. 'Zero-length' was defined as the amount of extension induced by a load of 0.5 g-wt.

A linear dependence of the response of spindle

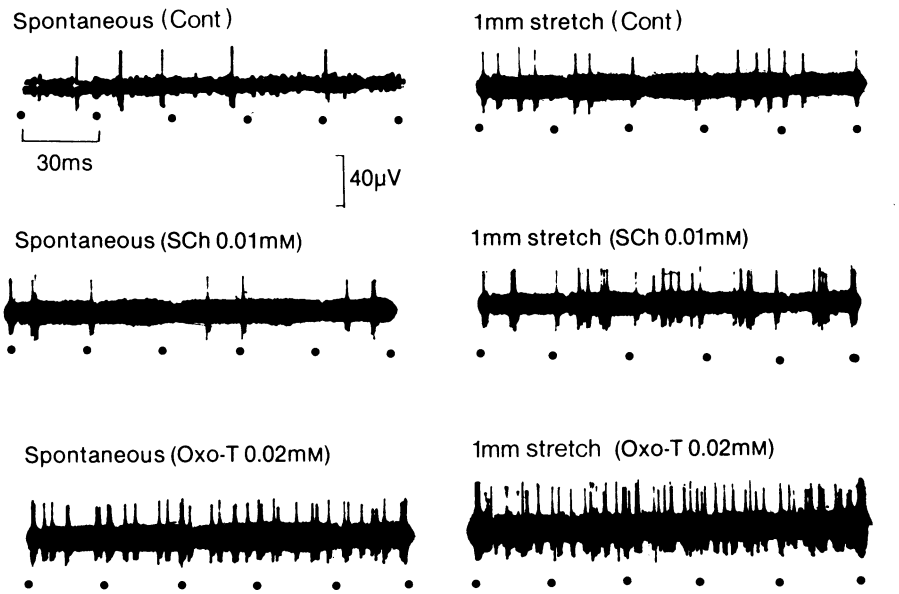

Figure 4 Typical oscilloscope records showing increase in rate of spontaneous (0 mm extension, left column) and stretch-induced (1 mm extension, right column) spindle afferent activity during the control period (Cont), in presence of succinylcholine (SCh.), and oxotremorine (Oxo-T).

afferent activity on muscle length was observed in most experiments. These results are pooled in Figure 3 (open circles). According to the anatomical evidence (Barstad *et al.*, 1965), about three muscle spindles are present in each hemidiaphragm of the rat. Therefore it is clear that our preparations often exhibited the characteristic discharge pattern of multifibre recordings since the whole phrenic nerve was employed for registration (see Methods). In order to allow comparison of all measurements, irrespective of the number of spindle afferents recorded in a given experiment, we have normalized the individual frequency values to the basic activity at 0 mm extension during the control period. This method was also used in Figure 5.

Effect of drugs

Physostigmine (2.5 to 20 μM) increased the rate of spontaneous and stretch-induced spindle afferent discharges (Figure 5). However, the extent of this increase varied considerably in different experiments.

Acetylcholine up to a concentration of 0.2 mM failed to alter the rate of spontaneous and stretch-induced afferent spindle discharges. But when administered in the bathing fluid after preincubation (10 min) with physostigmine (10 μM), acetylcholine caused a marked increase in the frequency of the afferent discharges and responses to stretch.

Atropine (0.005 to 0.1 mM), (+)-tubocurarine (1 to 4 μM) and hemicholinium-3 (0.1 to 0.4 mM) preincubated for a period of up to 160 min) failed to alter the rate of spontaneous spindle afferent discharges as well as stretch responses (Figure 5). Preincubation (10 min) with (+)-tubocurarine (2 μM), but not atropine (0.01 mM), inhibited the physostigmine-induced increase in rate of spindle afferent discharges and their response to stretch. The concentration of hemicholinium-3 used in this study (0.4 mM) has been found to produce complete inhibition of the extrafusal neuromuscular transmission in this preparation (Vedasiromoni & Ganguly, 1976; Das, Ganguly & Vedasiromoni, 1978).

Oxotremorine

Oxotremorine (0.005 to 0.02 mM), when added to the bathing fluid, markedly increased the rate of spontaneous and stretch-induced spindle afferent discharges (Figure 3, closed circles, and Figure 4). This effect of oxotremorine was an approximately linear function of its concentration. The onset was not immediate but occurred invariably between 2 to 3 min after its application, and the effect persisted usually

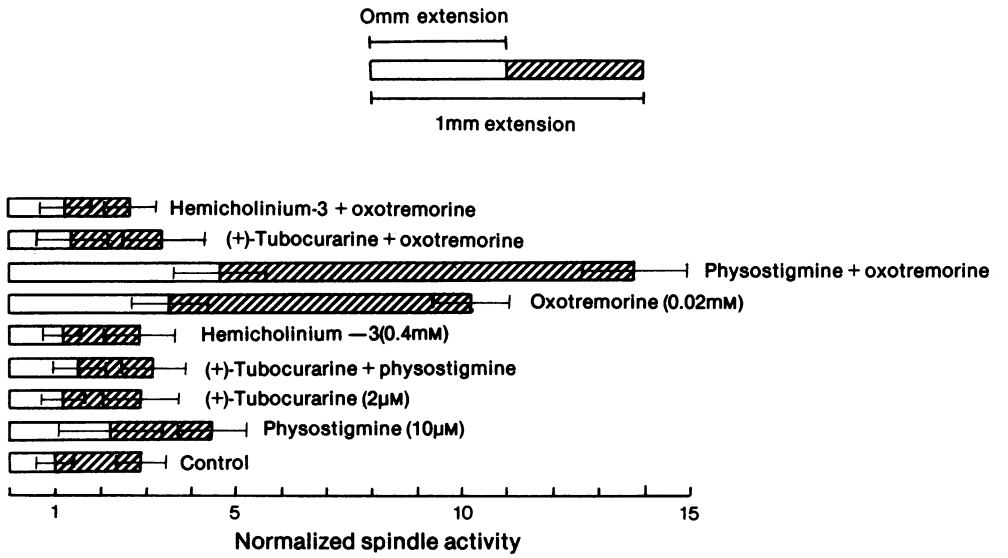

Figure 5 Summary of drug effects on muscle spindle afferent activity at 0 mm extension and 1 mm extension. All values of spindle afferent activity are normalized to the mean rate of discharge at 0 mm extension during the control period. Values are mean of at least six experiments; horizontal lines show s.e. means.

for more than 30 min, even after removal of the drug from the bathing fluid. This augmentation by oxotremorine of afferent discharges was further intensified by preincubation (10 min) with physostigmine (10 μM; Figure 5).

Preincubation with (+)-tubocurarine (2 μM for 10 min) or hemicholinium-3 (0.4 mM for 1 h) abolished the augmentation of spontaneous and stretch-induced spindle afferent discharges caused by oxotremorine (Figure 5). The spindle excitatory effect of oxotremorine remained unaltered in the presence of 0.1 mM atropine.

Discussion

This study demonstrates that the isolated phrenic nerve-diaphragm preparation of the rat may serve as a simple *in vitro* preparation for investigation of pharmacological aspects of proprioceptive activity in mammalian striated muscle. Cholinergic involvement in spindle afferent discharges including stretch responses was indicated by the augmentation produced by physostigmine and acetylcholine. The lack of effect of acetylcholine alone is presumably due to an abundance of cholinesterase present in the diaphragm muscle (Patterson, 1965). The cholinoceptors in the fusimotor system of rat diaphragm appear to be curariform in nature as the augmentation produced by acetylcholine and physostigmine was prevented by (+)-tubocurarine but not by atropine. The lack of influence of hemicholinium-3 and (+)-tubocurarine on spontaneous spindle afferent activity and stretch-induced responses excludes the role of acetylcholine in generation of sensory signals.

Unlike intact cat diaphragm (Corda *et al.*, 1965), no difficulty was encountered in differentiating the muscle spindle and Golgi tendon organ discharges in the present experiments. This is probably the result of elimination of interference by series coupled end organs from neighbouring parts of the musculature due to our method of dissection. The present experiments with oxotremorine confirm the recent observations of Fackler *et al.* (1977) on spindle afferent discharges in decerebrate cats and, in addition, identify the site and mechanism involved in this oxotremorine-action. An indirect mechanism, presumably excess release of acetylcholine, seems likely in the action of oxotremorine, since the effect was potentiated by physostigmine and prevented in the presence of hemicholinium-3 and (+)-tubocurarine.

The present study shows that oxotremorine is capable of causing a sustained increase in the afferent activity from muscle spindle endings. This observation is of interest since postural and motor abnormalities can be produced by affecting the function

of muscle spindles (for references, see Matsushita, 1964). It has recently been suggested that the spindle excitant effect of piperidine is involved in the mechanism of its production of motor abnormalities (Kidd & Kučera, 1977).

A cholinergic dominance at the skeletal myoneural apparatus including the fusimotor site and at the junction of motor axon collaterals to Renshaw cells in the spinal cord has been implicated in oxotremorine-induced motor disturbances (Ganguly & Chaudhuri, 1970; Ganguly, Ross, Haase & Cleveland, 1976; Fackler *et al.*, 1977; Das *et al.*, 1978). Clearly the oxotremorine-induced changes in spindle afferent discharge rate must affect motor performance independently of its effect on higher centres of the central nervous system, thus contributing further support for the aforementioned hypothesis. The common underlying mechanism at all these sites appears to be a presynaptic excess release of acetylcholine by oxotremorine. A massive release of acetylcholine from motor nerve terminals by oxotremorine has actually been demonstrated in the isolated phrenic nerve-diaphragm preparation of the rat (Das *et al.*, 1978).

The present results with oxotremorine are of interest in connection with the observations of Hagbarth, Hongell & Wallin (1970) that muscle spindle afferents are hyperactive in Parkinsonian patients.

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