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NEUROMUSCULAR TRANSMISSION IN ARTERIOLES OF GUINEA-PIG SUBMUCOSA

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SUMMARY

1. Intracellular recordings were made from arterioles lying in the submucosa of guinea-pig small intestine.

2. Low frequency perivascular nerve stimulation evoked subthreshold excitatory junction potentials which facilitated.

3. Higher frequency stimulation caused summation of excitatory junction potentials and the initiation of muscle action potentials.

4. Arteriolar constriction was only observed following the initiation of a muscle action potential.

INTRODUCTION

The majority of arterioles are innervated by adrenergic nerves which lie around the media (Norberg & Hamberger, 1964). Stimulation of these nerves causes arteriolar constriction in most arterial beds (Folkow & Neil, 1971). However there is little information about the sequence of electrical events, if any, which occur in the arteriolar smooth muscle cells during excitatory transmission.

Speden (1970) and Bell (1969) reported that sympathetic nerve stimulation initiated excitatory junction potentials (e.j.p.s) in arteries; under some circumstances the e.j.p.s evoked muscle action potentials. In contrast there are a number of reports that noradrenaline does not cause a change in membrane potential but can cause an increase in vascular smooth muscle tone (Somlyo & Somlyo, 1968; Casteels & Droogmans, 1976; Kitamura, Kuriyama & Sazuki, 1976). This has led to the suggestion that the neuronal control of vascular tone is not mediated by changes in membrane potential (Casteels & Droogmans, 1976).

In the present study, recordings have been made, *in vitro*, from the smooth muscle cells in the media of arterioles which distribute blood to the mucosa of guinea-pig small intestine, arterioles being defined as small blood vessels of external diameter 20–60 μm which originated from

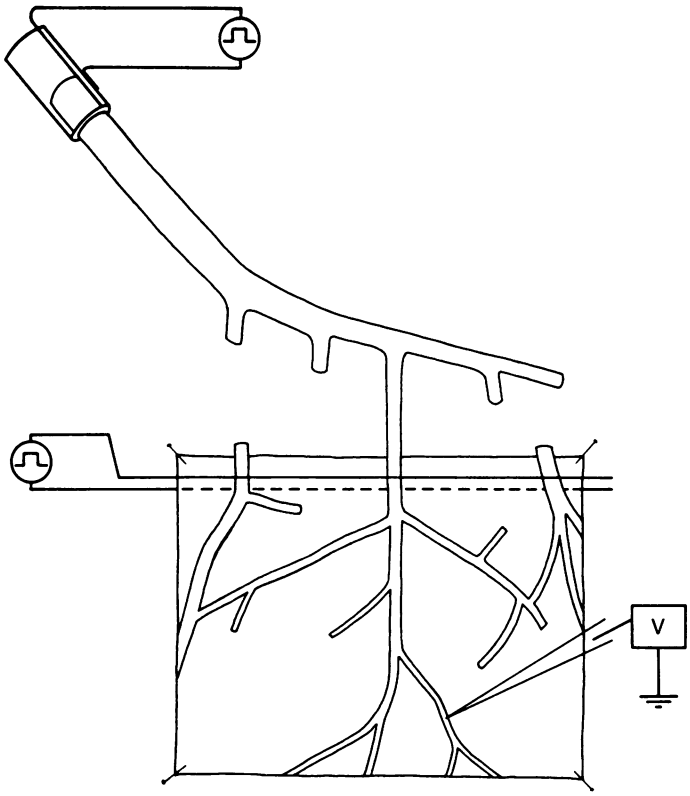
mesenteric distributing arteries. The mechanical responses of arterioles to periarterial nerve stimulation were observed visually during simultaneous intracellular recording from their smooth muscle cell layer. It was found that low frequency nerve stimulation evoked subthreshold excitatory junction potentials which were not associated with detectable changes in arteriolar diameter. Higher frequencies of stimulation led to facilitation and summation of e.j.p.s and the initiation of muscle action potentials. Arteriolar constriction could only be observed when a muscle action potential had been initiated. A preliminary report of these findings has been presented to the Australian Physiological and Pharmacological Society (Hirst, 1976).

METHODS

The preparation used in this study of neuromuscular transmission in arterioles was essentially the same as that described previously in reports of neurones of the submucous plexus of guinea-pig ileum (Hirst & McKirdy, 1975). The arterial network examined lies in the same connective tissue sheet as the submucous plexus and distributes blood to the mucosa (Lundgren, 1967). Preparations were made from segments of mid small intestine of guinea-pigs (male or female, 150–250 g). A segment of intestine (approximately 2 cm in length) complete with mesenteric arteries was pinned in a dissecting dish; the segment was opened by cutting along one side of the mesenteric border and then pinned flat with the mucosal surface uppermost. A strip of mucosa (5–6 mm wide, 1 cm long) was teased away on the edge adjacent to the intact mesenteric blood vessels. The preparation was inspected with the dissecting microscope and an arterial tree selected. The arteries and veins running in the mesentery were cut leaving only the artery supplying the selected arterial tree intact (see Text-fig. 1). The connective tissue sheet (containing arterioles and submucous plexus) was teased free of the circular muscle layer taking care not to stretch the artery connecting to the mesentery. The connective tissue was pinned in an organ bath (see Hirst, Holman & Spence, 1974), with the mucosal surface uppermost and viewed with an inverted compound microscope (Reichart Biovert, magnification $\times 40$–630). The preparation was stimulated either by drawing the mesenteric artery into a suction electrode (see Text-fig. 1) or by use of a pair of transmural electrodes (see Hirst *et al.* 1974) positioned near the main arteriolar trunk (see Text-fig. 1). Physiological solution (composition (mM); NaCl 120; KCl 5; $CaCl_2$ 2·5; $MgCl_2$ 2; $NaHCO_3$ 25; NaH_2PO_4 1; glucose 11, gassed with 95% O_2:5% CO_2) at 35 °C flowed continuously through the recording chamber at 2 bath volumes/min.

Intracellular recordings were made from arterioles using micro-electrodes filled with 2 M-KCl. Impalements of 1–2 hr could only be maintained by using high resistance electrodes (120–200 MΩ) pulled to have a long shank (1–1·5 cm) from Micropet glass (Clay Adams, 20λ). These electrodes were not dislodged by arteriolar constriction, rather the micro-electrode tip region appeared to move with the arterial wall. Membrane potentials were recorded using conventional methods. In some experiments averaged responses to nerve stimulation were obtained by use of a Biomac 1000.

In seven experiments, preparations were made from segments of ileum which had been extrinsically denervated by freezing three adjacent mesenteric arcades 7 days previously (Gabella & Juorio, 1975).



Text-fig. 1. Schematic diagram of arteriolar tree preparation used in these experiments. A branch of the mesenteric artery, drawn into a suction electrode, leads to the submucosa and divides into small arterioles. The preparations of submucosa were pinned on a cover-slip and viewed with a microscope (for further details see Methods).

RESULTS

General observations. Low power microscopic examination ($\times 40$) of the preparations revealed a branched network of arterioles arising from the artery lying in the mesentery. The mesenteric arteries at their points of entry into the small intestine usually had external diameters of 60–120 μm; branches of varying size led from these arteries and ran along the connective tissue sheet. After subsequent branching most of the smaller arterioles (diameter 20–30 μm) ended abruptly: their true terminations presumably lying in the dissected away mucosa. A proportion of arterioles (diameter 30–40 μm), anastomosed with similar sized branches of either an adjacent arteriolar tree or with the arteriolar tree which originated from the opposite border of the preparation, that is, in the

intact gut they would form a complete loop. Using higher power magnification (× 160–630) both the arteriole walls and any red blood cells within them could be visualized (see Pl. 1*A*).

The majority of preparations showed no detectable mechanical activity in the absence of nerve stimulation. In a small proportion (less than 5%), arterioles were rhythmically active; a wave of contraction would originate at a point in the arteriolar tree and spread for a few hundred microns. In such preparations activity was confined to a small region and on no occasion was an entire tree spontaneously active. On most occasions these spontaneous movements lasted for less than 30 min and then the entire preparation became quiescent. In two preparations (less than 1% of the total preparations) spontaneous mechanical activity persisted for longer than 3 hr. It is not known whether the spontaneous activity results from damage during the dissection or whether such activity might be normal behaviour of these preparations *in vivo*.

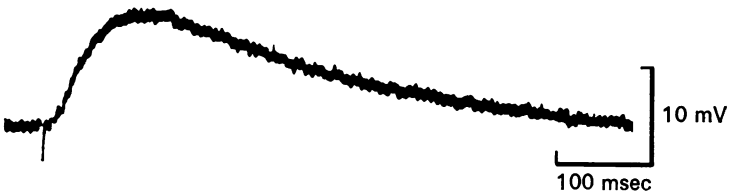
Mechanical activity could be evoked either by stimulating the main artery (perivascular stimulation) with trains of brief stimuli (train duration 0·5–1 sec; stimulus frequency 5–20 Hz, pulse width 0·05–1 msec), or by applying similar trains of stimuli to the transmural electrodes (transmural stimulation). Mechanical responses could usually be evoked at regular intervals for several hours although in some preparations the responses failed after 1–2 hr.

The responses produced by trains of stimuli varied depending upon the diameter of the arteriole. The small arterioles appeared to close almost completely whereas the larger arterioles (greater than 30 μm) constricted but were not completely occluded (see Pl. 1*A*, *B*). During the contraction red blood cells moved in the direction of the main artery. As the arterioles relaxed the red blood cells flowed back into the arterioles. Since the arteriolar tree was open at both the central and peripheral ends, the retrograde movement of red blood cells can be explained by an uneven distribution of resistance in the arteriolar tree; the smaller more peripheral arterioles presenting the higher resistance.

During repetitive low frequency (1 Hz) perivascular stimulation no general change in arteriole diameter was detected, rather, on a few occasions, small arterioles closed and relaxed intermittently. Although it will be appreciated that *in vivo* the arterial bed is perfused with blood under pressure and such closures would not be expected to occur, the intermittent generation of tension in part of the bed might contribute to peripheral resistance.

On no occasion have movements of the venules which lie close to the main arterioles been observed, either spontaneously or as a result of perivascular stimulation.

Electrical properties. Intracellular recordings were made from arterioles which were clearly visible when viewed under high magnification (× 160–650). Impalement of an arteriole was associated with a negative deflexion (40–75 mV) on the voltage record. When values less than 65 mV were recorded, the membrane potential decreased over the next few minutes. However, stable records of membrane potentials greater than 65 mV lasting up to 3 hr have obtained from quiescent preparations. During these periods subthreshold electrical activity, either myogenic or neurogenic, was not detected. When inward or outward currents of low intensity (10^{-11}–10^{-10} A) were injected into an impaled arteriolar muscle cell via the recording electrode, membrane potential changes caused by these currents were less than 5 mV. This would suggest an upper limit of input resistance of 50 MΩ. More intense currents than these caused

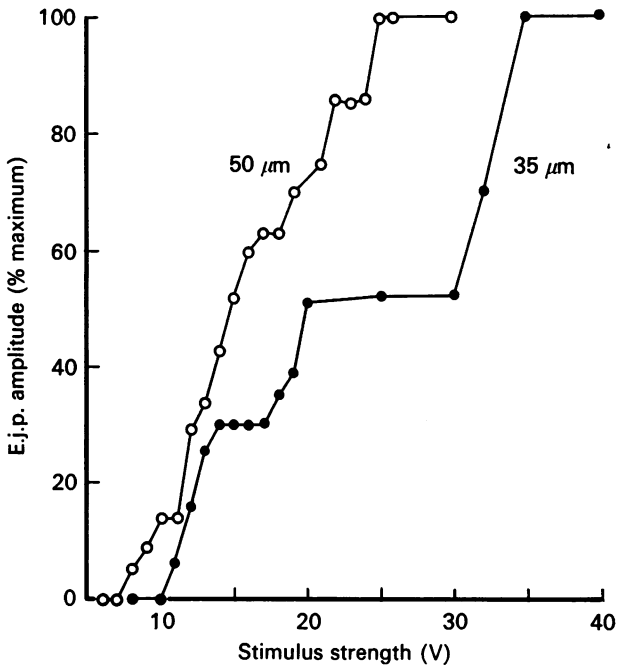


Text-fig. 2. Excitatory junction potential recorded from an arterial in response to supramaximal transmural stimulation. The record was taken during a train of stimuli (0·5 Hz) and is therefore a facilitated response. This response and others recorded from other preparations were not associated with a change in arteriolar diameter.

changes in micro-electrode resistance and prevented an accurate determination of input resistance. Even large intensity depolarizing currents (up to 1×10^{-9} A) failed to initiate muscle action potentials. These observations would suggest that arteriolar smooth muscle cells like those of arteries and other smooth muscles are electrically interconnected (Mekata, 1974, 1976; Tomita, 1970). The failure to initiate action potentials by point depolarization could in part be explained by syncitial couplings and perhaps because of a high threshold for the initiation of action potentials (see for example Holman, Taylor & Tomita, 1977).

Responses evoked either by periarterial or transmural stimulation; low frequency stimulation. A brief stimulus (pulse width 0·05–0·5 msec stimulus voltage > 20 V) applied either to the transmural or to the periarterial stimulating electrode evoked an excitatory junction potential (e.j.p.) in all arterioles examined. E.j.p.s had amplitudes of some 2–6 mV which rapidly increased to 10–15 mV during repetitive low frequency stimulation (0·2–0·5 Hz). An example of an e.j.p. recorded during a train of stimuli (0·5 Hz) is shown in Text-fig. 2. The time to peak potential was 110 msec (S.E. of mean ± 4 msec, n = 18) and the time from half-peak amplitude

on the rising phase to half-peak amplitude on the falling phase (half-width) was 349 msec (S.E. of mean $\pm$ 22 msec, n = 18, each n value was obtained from automatically averaged e.j.p.s using a minimum of sixteen sweeps). In most records although successive e.j.p.s fluctuated in amplitude (Text-fig. 5), their time course showed little fluctuation. In a few records, during a train of stimuli, an e.j.p. of briefer rise time than most others



Text-fig. 3. Relationships between stimulus strength and amplitude of excitatory junction potentials from two separate arterioles. Each point is the averaged response of sixteen successive stimuli. Stimulus strength response curves were obtained by first determining the supramaximal stimulus strength and reducing the stimulus voltage in increments. The voltage scale measures voltage setting on the stimulator output. Note that in each curve there are a series of plateaux which are assumed to correspond to recruitment of additional sympathetic fibres. The diameters of the two arterioles were 50 μm (open circles) and 35 μm (filled circles).

recorded from that cell, was observed (see for an example Text-fig. 4). Excitatory junction potentials had identical amplitudes and time course whether initiated by periarterial or by transmural stimulation. The only difference between records taken during a given impalement was that the latency of e.j.p. evoked by periarterial stimulation was some 30–40 msec

longer than that of an e.j.p. (10–15 msec) recorded close to the transmural stimulating electrodes. Since these electrodes were separated by 1 cm, the conduction velocity of a nerve action potential in periarterial axons must be of the order of 0·3 m/sec.

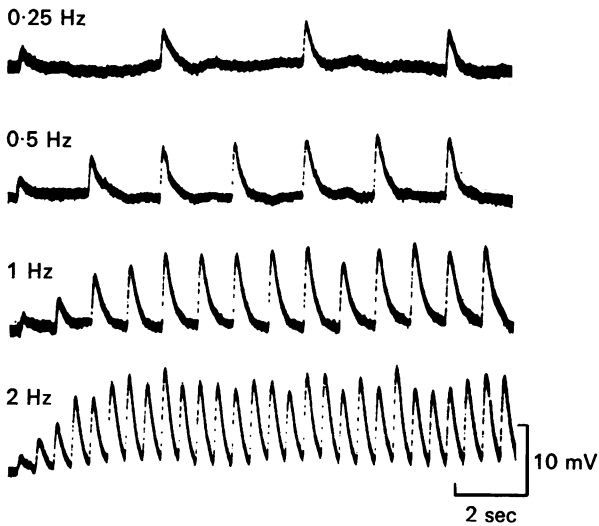
In an attempt to determine the number of fibres that functionally innervate an arteriole, the stimulus strength was varied. Records were made from arterioles of external diameter greater than 30 μm as it was easier to obtain prolonged penetrations from such vessels. The preparation was stimulated repetitively at 1 Hz, and the amplitude of the average of sixteen successive e.j.p.s determined. Subsequently the stimulus strength was varied and a new average determined. All stimulus–amplitude curves were determined by first finding the maximum stimulus strength and then reducing the stimulus strength so as to avoid complications due to recruitment of unfacilitated terminals (Bell, 1969). The results from two experiments are shown in Text-fig. 3. Assuming that each plateau on the curve represents recruitment of distinct nerves it can be seen that the larger arteriole was innervated by four nerves; the smaller by three nerves. This would agree with histological finding that arterioles of this diameter are innervated by two to six fluorescent adrenergic nerve fibres (Furness, 1973). However, it is unlikely that each individual smooth muscle cell is innervated directly by three or four nerves, rather these observations support the idea that the smooth muscle cells are coupled together electrically and suggest that the resulting syncitium is innervated by three or four fibres. It is not surprising, therefore, that successive e.j.p.s fluctuated in amplitude, as such a small number of fibres innervate the syncitium.

During trains of stimuli, it was occasionally possible to detect small depolarizing potentials (0·5–1·2 mV) of variable latency. Since repetitive nerve stimulation has been shown to increase the rate of spontaneous transmitter release (for a review see Hubbard, 1973), it seems likely that these potentials reflect the 'spontaneous' release of quanta of transmitter (see also Bell, 1969). The time course of these miniature excitatory junction potentials was only slightly more rapid than that of the evoked potentials (see Text-fig. 4).

High frequency stimulation. Successive e.j.p.s evoked by trains of supramaximal stimuli increased in amplitude (Text-fig. 5). At frequencies as low as 0·1 Hz, successive e.j.p.s were facilitated; facilitation at higher frequencies became more marked. In three experiments, preparations were stimulated with pairs of impulses each 50 sec with intervals between the pulses in the range 1–16 sec. Averages of eight responses at each separation (1, 2, 4, 6, 8, 10, 12, 14 and 16 sec) were taken. The decay of facilitation obtained by plotting the ratio of test to conditioning e.j.p.

amplitude could be described in each case by a single exponential with time constants of decay of 5, 4 and 5·5 sec. These values are similar to those reported by Bennett (1973) from studies on transmission at the

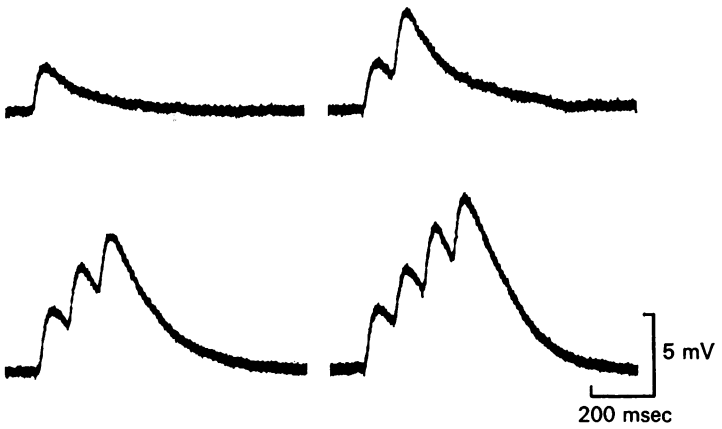
Text-fig. 4. 'Spontaneous' e.j.p.s recorded from an arteriole (diameter 25 μm) during repetitive submaximal stimulation (stimulation frequency 1 Hz). Small amplitude depolarizations which are not synchronized with the evoked potentials can be seen. Note also the variation in amplitude and time course of successive evoked junction potentials.



Text-fig. 5. Facilitation of excitatory junction potentials during trains of stimuli at varying frequencies. Facilitation becomes more marked as the stimulus frequency was increased until at frequencies of 1–2 Hz the facilitated e.j.p. was some tenfold larger in amplitude than the initial junction potential. Calibration bars apply to each record.

mouse vas deferens. The long time constant of decay of facilitation would suggest that *in vivo*, where many sympathetic post-ganglionic fibres are tonically active (Skok, 1973), many sympathetic pre-junctional terminals will continually be in a facilitated state.

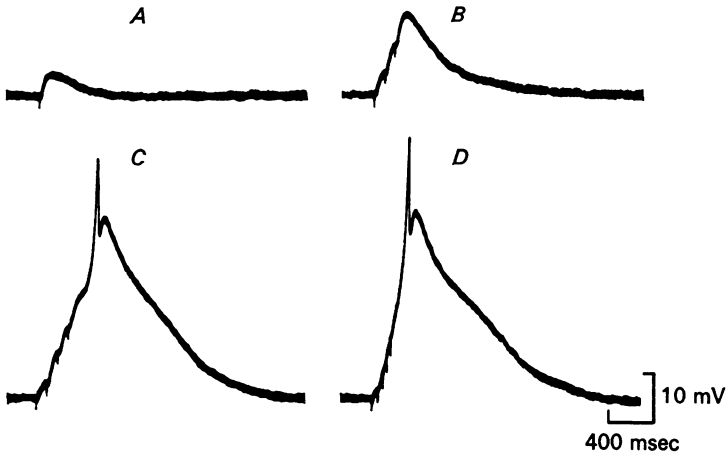
Initiation of muscle action potentials. At frequencies of stimulation above 1 Hz, successive e.j.p.s summed to give large amplitude subthreshold



Text-fig. 6. Summation of excitatory junction potentials. During short trains of stimuli (stimulus frequency 5 Hz) successive e.j.p.s summed to give subthreshold depolarizations which were not associated with muscle movement. The rate of decay of potential was similar after both small and large potential changes. Calibration bars apply to each record.

potentials (Text-fig. 6) which were not associated with any visually detectable movement in the region of arteriole close to the recording electrode. However, if the stimulation frequency or the train duration was increased the e.j.p. complex further increased in amplitude and initiated a muscle action potential (Text-fig. 7). Visual observation of the preparation now indicated that the arteriole had constricted (see Pl. 1). A further increase in stimulation frequency shortened the delay before the initiation of an action potential and on many occasions caused an increase in its amplitude. Muscle action potentials usually had a rapid rise time, brief duration and then, a second slower component on the decay (see Text-fig. 7*C* and *D*). In some preparations the initial component was completely absent and only the slower second component could be detected. Invariably if the second component alone could be detected, arteriolar constriction could be observed. The second component preceded the onset of visible movement. Further studies to clarify the time course, the variable amplitude of action potentials and the underlying permeability changes responsible for the action potential are required but it would

only be possible to analyse these factors if action potentials could be evoked by direct stimulation. Since the second component of the action potential alone consistently evoked constriction this component may be like that in cardiac muscle and involve a calcium current (Reuter, 1973).



Text-fig. 7 (*A*, *B*, *C* and *D*). Initiation of muscle action potentials by arterial nerve stimulation. In the records shown in Text-fig. 7*A*, *B* and *C*, periarterial nerves were stimulated with a single stimulus (*A*), three stimuli (*B*), and four stimuli (*C*); train frequency for *B* and *C* was 10 Hz. In record *D*, five stimuli were applied, train frequency 20 Hz. Note that the action potentials initiated in *C* and *D* had different latencies from the onset of stimulation and their amplitudes were dissimilar. Visual observation of the preparation indicated that arteriolar constriction occurred but only during the time that records *C* and *D* were obtained. Calibration bars apply to each record.

Effect of extrinsic denervation. Excitatory junction potentials could not be detected when recordings were made from arterioles which had been extrinsically denervated by prior freezing of mesenteric arteries. Since this procedure causes a loss of fluorescence around mesenteric arterioles (D. Costa, personnel communication) and a loss of noradrenaline in these tissues (B. Jarrott & G. D. S. Hirst, unpublished observations), it seems likely that e.j.p.s result from the release of noradrenaline from post-ganglionic sympathetic axons. No electrophysiological evidence was obtained to support the view that these arterioles receive an intrinsic inhibitory innervation (Biber, Fara & Lundgren, 1973).

DISCUSSION

Excitatory junction potentials were recorded from arterioles in response to sympathetic nerve stimulation. Each arteriole was innervated by only a few nerve fibres (three to six); simultaneous stimulation of all of these fibres only evoked a subthreshold e.j.p. Visual observation of the preparation indicated that such a potential did not cause arteriolar constriction. Arteriolar constriction was evoked by repetitive nerve stimulation but only if successive e.j.p.s summed and initiated a muscle action potential. These observations suggest that, *in vivo*, changes in peripheral resistance might result only from tonic activity in many sympathetic post-ganglionic fibres producing a summed post-junctional depolarization.

In most respects, neuromuscular transmission in arterioles resembled transmission in the vas deferens, a preparation where excitatory sympathetic transmission has been examined (Burnstock & Holman, 1961; Bennett, 1972). Excitatory junction potentials had time courses similar to those recorded from the guinea-pig vas deferens (Burnstock & Holman, 1961). Like the vas deferens, successive e.j.p.s facilitated (Burnstock, Holman & Kuriyama, 1964) and the facilitation decayed with a long time constant (Bennett, 1973). Because spontaneous small amplitude excitatory junction potentials were occasionally detected, it is likely that like other junctions, transmitter is released in quanta (Katz, 1969). However the spontaneous junction potentials were not dramatically different in time course from evoked potentials; this is not the case for the vas deferens (Tomita, 1967). It is possible that the electrical properties of the two preparations are dissimilar.

If the excitatory junction potentials recorded from arterioles result from the release of noradrenaline from sympathetic nerve varicosities, these observations are at variance with the reports that noradrenaline does not cause a change in vascular smooth muscle membrane potential (Somlyo & Somlyo, 1968; Casteels & Droogmans, 1976). Furthermore, since arteriolar constriction could only be detected after initiation of an action potential, the observations reported here do not support the idea that the neuronal control of vascular smooth muscle tone is mediated by a mechanism which does not involve a change in muscle membrane potential (Casteels & Droogmans, 1976).

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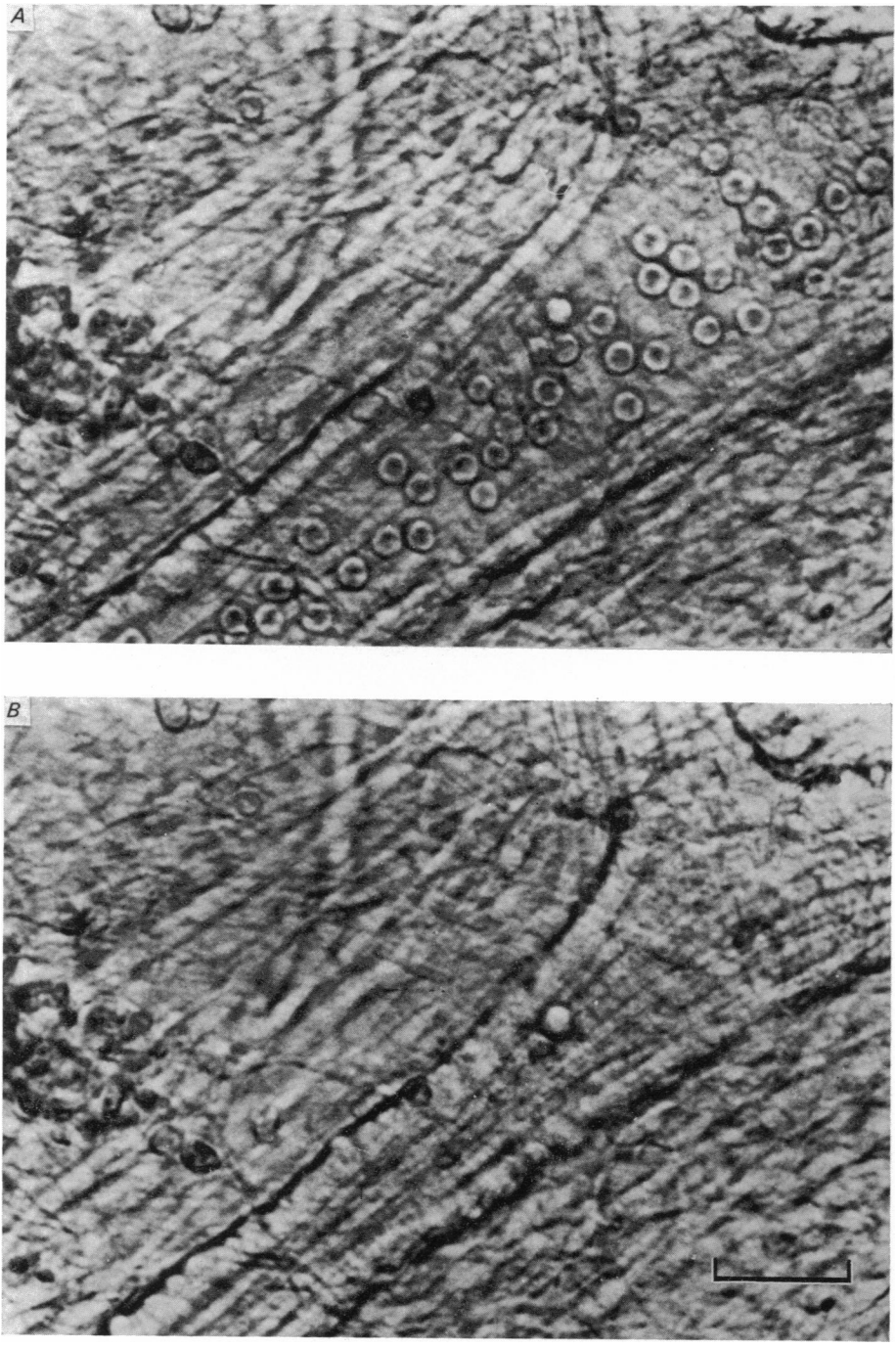

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EXPLANATION OF PLATE

A, *B*, a living arteriole viewed with conventional optics. Pl. 1 *A* shows relaxed arteriole of fairly large diameter (50 μm), running diagonally across the plate; a finer arteriolar branch at the arterial end of the arteriole (upper right) is visible. Pl. 1 *B* shows the same portion of arteriolar tree but was photographed during a period of repetitive nerve stimulation (5 sec at 10 Hz); the exposure occupied the final second of the stimulus train. Note that the arteriole diameter has decreased and that the red blood cells visible in the relaxed arteriole (Pl. 1 *A*) have been expelled. Shortly after the end of the train of stimuli, the arteriole relaxed and returned to its former shape. Calibration bar of 30 μm applies to each Plate.