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Electrical Changes Underlying Excitation and Inhibition in Intestinal and Related Smooth Muscle

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III. THE MECHANISMS OF ACTION OF NEUROTRANSMITTERS

Electrical changes underlying excitation and inhibition in intestinal and related smooth muscle

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[Plate 12]

Intestinal smooth muscle is normally spontaneously active and contraction is associated with spike activity. Stimulation of excitatory (cholinergic) nerves increases spike frequency while inhibitory (adrenergic) nerve activity reduces slow waves and spikes without necessarily producing hyperpolarization. Activity of intrinsic nerves produces inhibition with marked hyperpolarization.

The anococcygeus muscle of the rat, a muscle associated with the alimentary canal, has a dense adrenergic innervation and has neither resting tone nor spontaneous activity. The mean resting potential is 58.4 mV. Field stimulation produces graded depolarization associated with contraction and abolished by phentolamine. The depolarization has an initial component of up to 10 mV followed by a response which can reach 50 mV, the largest sometimes having a single spike on the rising phase. Application of noradrenaline or guanethidine produces depolarization with oscillations at 1/s and maintained contraction. Field stimulation at low frequencies during this contraction causes relaxation and reduction in the membrane oscillations but no repolarization.

Electrophysiological studies have been made on several regions of the alimentary canal and while some local variations do occur all have several properties in common. The first of these is the presence of spontaneous electrical and mechanical activity. Figure 1 shows electrical records from three different cells in the rabbit colon together with the mechanical activity of the tissue. The membrane potential was not stable but slow waves of depolarization culminated in one or more spike potentials, each coinciding with increase in tension.

Most regions of the alimentary canal possess both motor and inhibitory extrinsic innervation corresponding to parasympathetic and sympathetic nerves. In addition, the alimentary canal is unique in possessing in its intrinsic plexuses neurons entirely confined within its walls; these neurons are synaptically related to one another and organized for reflex activity. An architectural basis for intrinsic inhibitory and excitatory neurons on the efferent side of intrinsic reflex arcs therefore exists and there is experimental evidence for the existence of at least the inhibitory neuron.

The mechanical effect of parasympathetic nerve activity and its electrical basis is shown in figure 2 for stimulation of the pelvic nerve to the colon in the rabbit. At low frequencies each stimulus caused an excitatory junction potential some of which culminated in spike potentials. The increase in spike potential frequency caused increase in tension. Slightly raising the stimulus frequency increased the amplitude of the junction potential and the frequency of spikes with an increase in tension. When the frequency of stimulation was gradually raised from 1 to 12/s a maintained stable depolarization was eventually produced in which the spike potentials were replaced first by a damped oscillation and finally by a maintained stable depolarization

(figure 2*d*). Yet in spite of the absence of spike potentials, contraction remained maximum. Similar results have been obtained from stimulation of the pelvic nerve to both guinea-pig and rabbit colon (Furness 1969).

The electrical basis of the response to stimulation of the extrinsic sympathetic nerves has also been studied (Gillespie 1962*a*; Bennett, Burnstock & Holman 1966*a*). Two mechanisms have

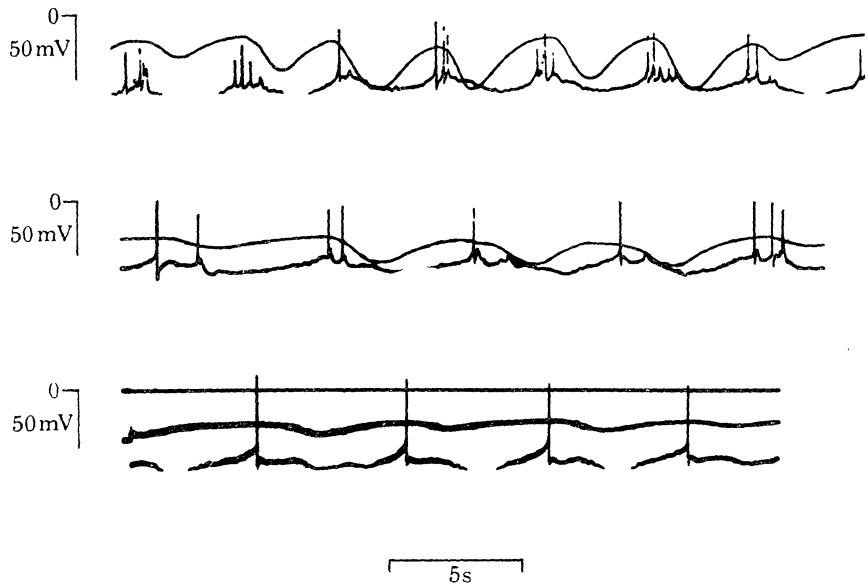

FIGURE 1. Spontaneous mechanical (upper) and electrical activity of the rabbit colon. In these records an increase in tension is shown downwards (Gillespie 1962*a*).

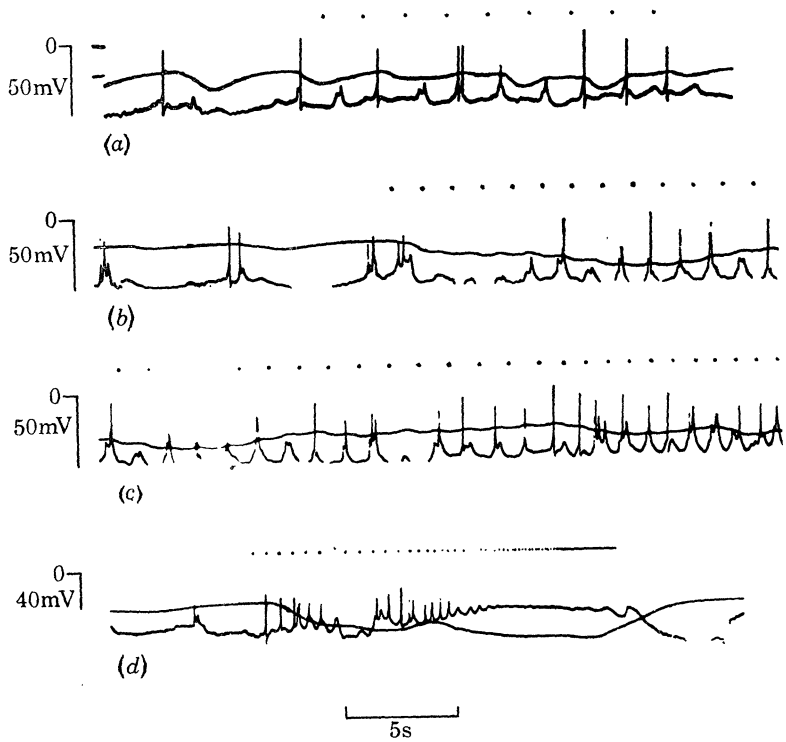

FIGURE 2. Electrical and mechanical responses of the rabbit colon to parasympathetic nerve stimulation. Each stimulus is indicated by a black dot above the record (Gillespie 1962*b*).

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been suggested as contributing to the mechanical inhibition; first abolition of the slow spontaneous waves of depolarization; secondly, the production of hyperpolarization. The first of these is shown in the upper part of figure 3. These five records are from the same cell and show the effect of stimulating the extrinsic sympathetic nerves at different frequencies. The top record shows the normal spontaneous electrical and mechanical activity. Unlike parasympathetic nerve stimulation single stimuli to the sympathetic nerve are never effective in producing a mechanical response or any change in the membrane potential. Stimulation at 5 and 10/s in the next two records slowed the rate of depolarization of the slow waves and therefore diminished the frequency of the spike bursts with a resultant reduction in tension. Higher frequencies of stimulation completely abolished all pacemaker activity but without evidence of hyperpolarization. Hyperpolarization was seen if the muscle was first depolarized by stretch (figure 3*f*). Stretch alone caused a continuous discharge of spike potentials at an average frequency of 1/s, and sympathetic nerve stimulation caused both abolition of these spike potentials and hyperpolarization of the membrane which outlasted the stimulus. As the muscle again depolarized spikes reappeared and the first of these preceded the reappearance

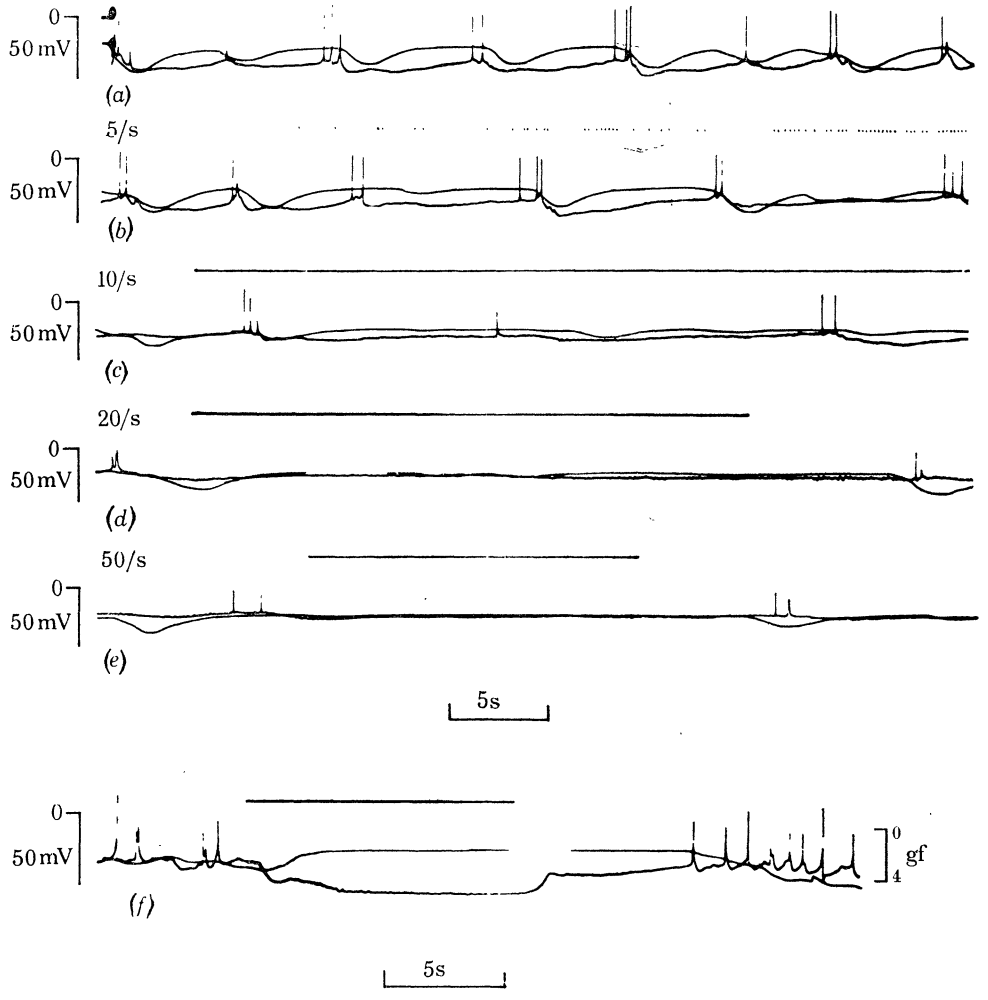

FIGURE 3. Inhibitory responses to stimulation of sympathetic nerves at 5, 10, 20 and 50/s (*b*, *c*, *d*, *e*). (*a*) shows spontaneous activity in the absence of stimulation. In (*f*) the muscle was depolarized by stretch and stimulated at 50/s (Gillespie 1962*a*).

of mechanical contraction. In the taenia coli essentially similar results were observed on stimulating the extrinsic sympathetic perivascular nerves. The taenia coli, a much thinner muscle than the rabbit colon, is usually mounted under several grams-force tension and discharges spike potentials continuously. In these circumstances hyperpolarization is a more noticeable aspect of the electrical response (Bennett *et al.* 1966*a*).

The existence of inhibitory neurons in the gut wall was suggested by Langley (1898) and pharmacological evidence was offered by Ambache (1951) and others but the first electrophysiological evidence for their presence in the taenia coli was published by Burnstock, Campbell, Bennett & Holman (1963). They have since been reported in the colon of both guinea-pig

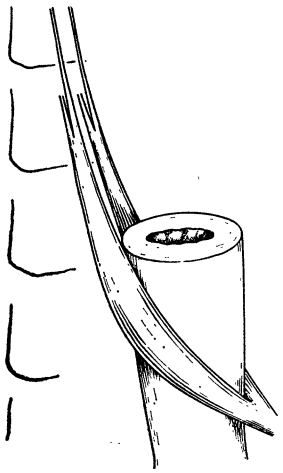

FIGURE 4. The position of the anococcygeus muscles in the male rat. The muscles pass from the coccygeal vertebrae to the ventral surface of the colon with extensions to the retractor penis muscles.

and rabbit (Furness 1969, 1970). The characteristics of the response to stimulation of these nerves are quite unlike those of the response to sympathetic nerve stimulation. A single stimulus is effective; the response is a hyperpolarization which may be as large as 25 mV for a single stimulus and 35 mV for repetitive stimulation; maximum responses require only low frequency stimulation of about 5/s and both hyperpolarization and the mechanical inhibition it induces rapidly 'escape' if stimulation is continued (Bennett, Burnstock & Holman 1966*b*). Guanethidine and bretylium which block the extrinsic sympathetic nerves are ineffective in blocking the response to stimulation of these intrinsic neurons.

Transmural stimulation will also excite excitatory neurons (Bennett 1966; Gillespie & Mack 1968), but the properties of the response do not differ, other than in latent period, from the response to stimulation of the extrinsic parasympathetic nerves and they may therefore be or include the postganglionic parasympathetic neurons. The relationship of the intrinsic inhibitory neuron to the extrinsic innervation is obscure. Burnstock, Campbell & Rand (1966) stimulating an adjacent flap of caecum have shown that inhibition can be produced in the adjoined taenia coli and that this effect is at least partially abolished by ganglion blocking agents. The older literature has many references to inhibition, particularly in the stomach, produced by vagal stimulation. Such indirect evidence has suggested that the intrinsic inhibitory neuron lies in the efferent vagal pathway. Furness (1969), on the other hand, has been unable to find any connexion between the pelvic parasympathetic pathway and the inhibitory neurons in the colon.

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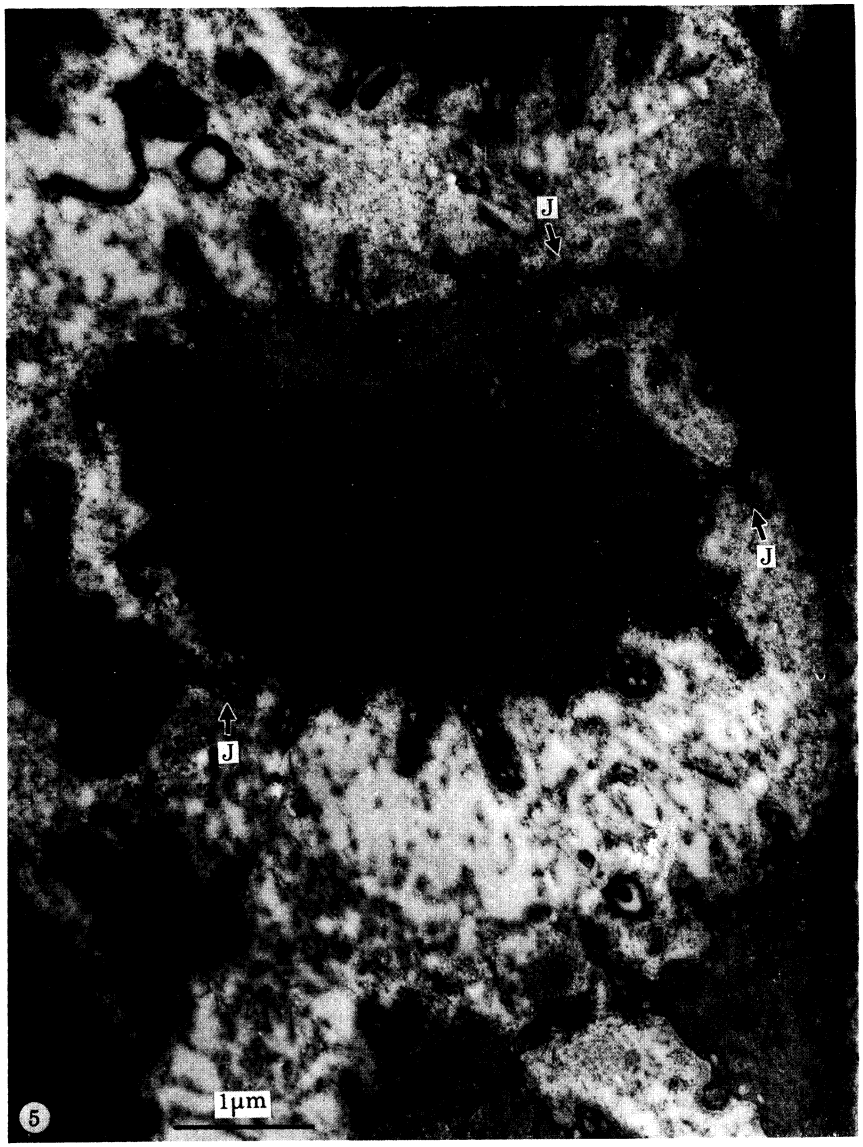

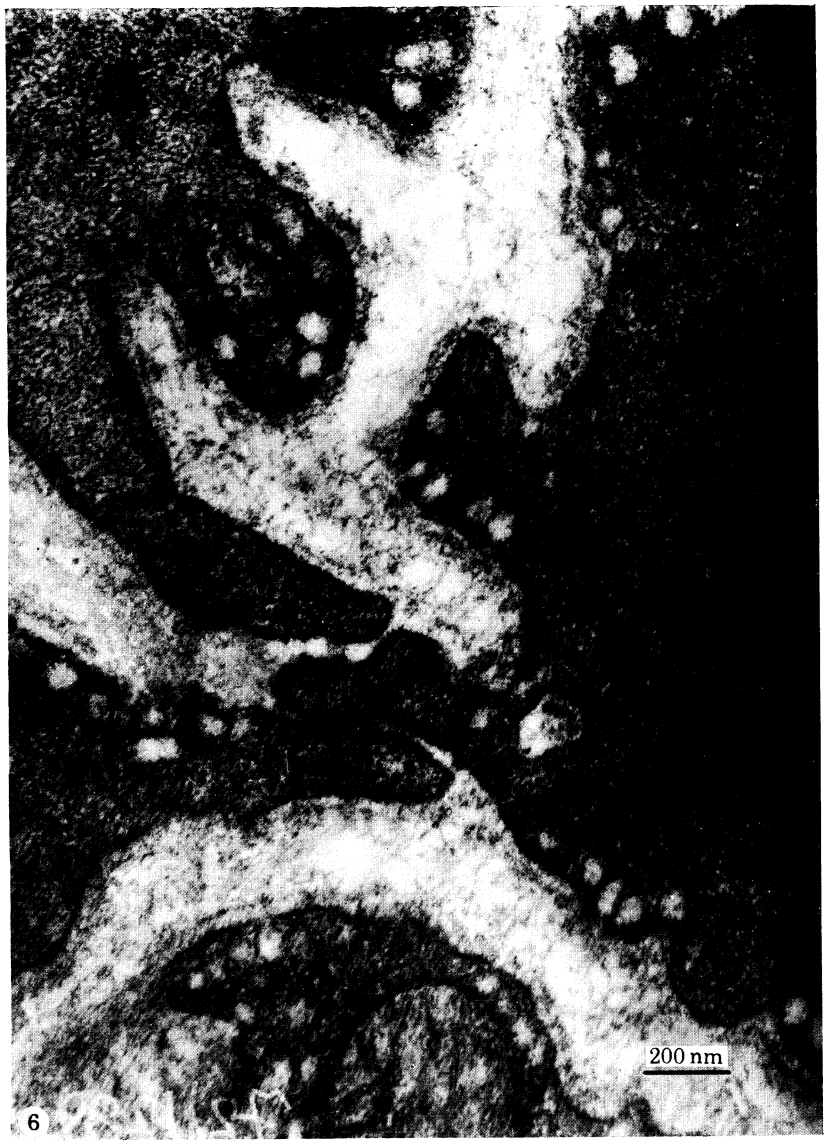

FIGURE 5. Transverse section of the anococcygeus muscle showing a single smooth muscle cell sectioned at the level of the nucleus, with several neighbouring cells. Caveolae, surface projections and close junctions (J) with two other cells are visible.

FIGURE 6. Close junctions between three neighbouring cells, with a minimum separation of 14 nm. Basement membrane is present in the gap.

(*Facing p. 99*)

What we now propose to describe is a muscle associated with the alimentary canal, but whose properties are in almost every respect the opposite of those just described. The muscle is the anococcygeus in the rat. It is paired, and arises in the pelvis from the coccygeal vertebrae by a true tendonous origin (figure 4). Lying just behind the colon, the two muscles pass on either side to form a ventral bar from which, in the male, fibres are given off to form the retractor penis muscle. In the female the ventral bar is poorly developed. The muscles are about 3 cm long by 0.5 cm broad and 250 to 300 μm thick. The muscle, unlike the alimentary canal, has a dense adrenergic innervation uniformly distributed throughout. Treatment by the Falck fluorescence technique showed bright green adrenergic nerves among the non-fluorescent smooth muscle (Gillespie & Maxwell 1971).

The ultrastructure of this muscle was examined in material fixed in 2 % glutaraldehyde for 18 h, postfixed in 1 % osmic acid and stained with uranyl acetate. The muscle cells were characterized by numerous surface projections, a profusion of caveolae and particularly by numerous close junctions with neighbouring cells. Figure 5, plate 12, shows these features with a central cell of 5 μm maximal diameter sectioned transversely in the region of the nucleus and with several neighbouring cells two of which establish close junctions with the central cell. Figure 6, plate 12, shows at higher magnification a close junction between the processes of three cells. The minimum gap is only 14 nm but even so it is filled with basement membrane. We have never seen plasma membrane fusion nor any specialization of the membranes on either side of the junction.

Nerves are easily found among the muscle cells and consist of usually two to five unmyelinated fibres many in Schwann sheaths. Occasionally single fibres and complexes of up to 20 fibres are seen. We have never found any close synaptic relation between muscle and nerve. In the caudal part of the muscle, where the extrinsic nerve enters, larger bundles of nerve fibres surrounded by a well-defined perineurium were found. These bundles, which were found both on the surface and within the muscle, contained several large (8 to 10 μm) myelinated fibres as well as numerous unmyelinated fibres.

This muscle can be isolated *in vitro* and stimulated either by field stimulation or by drugs. Tension was recorded isometrically by attachment to a tension transducer. The tissue was stimulated by electrical pulses delivered through two silver–silver chloride ring electrodes around the tissue. 1 ms pulses at supramaximal voltage were used. Drugs were added to the perfusion fluid. The effects of electrical stimulation were due to activity of intramural nerve fibres since drugs which block adrenergic nerves completely abolished both the mechanical and electrical excitatory response of the muscle cells.

The muscle was completely lacking in tone or rhythmic activity but contracted powerfully to field stimulation, to noradrenaline or to muscarinic drugs such as furmethide (figure 7). The motor response to field stimulation was abolished by phentolamine as was that to noradrenaline. The muscle also possessed a powerful inhibitory innervation. This could only be demonstrated if the tone of the muscle was first raised. Guanethidine was an excellent drug for this purpose since it acted as an indirect sympathomimetic to raise tone and at the same time blocked presynaptically the motor response. Figure 8 shows first the effect of field stimulation before guanethidine; the addition of guanethidine caused a rise in tone and field stimulation then caused large inhibitory responses, unaffected by hexamethonium but blocked by tetrodotoxin. We have not so far been able to identify the inhibitory transmitter though several possible transmitters have been investigated (Gillespie 1972).

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We would now like to describe the electrical basis of the motor and inhibitory response but first, one other mechanical record requires description (figure 9). If the tissue was stimulated at very low frequencies, one every 10 s, then each stimulus produced a discreet mechanical response which failed to summate though there was some potentiation towards the end of the train. At a slightly higher frequency of one every 5 s these small mechanical responses were close enough to summate but in addition at a certain point there was a sudden increase in the mechanical increment. At still higher frequencies this step increase in the mechanical increment

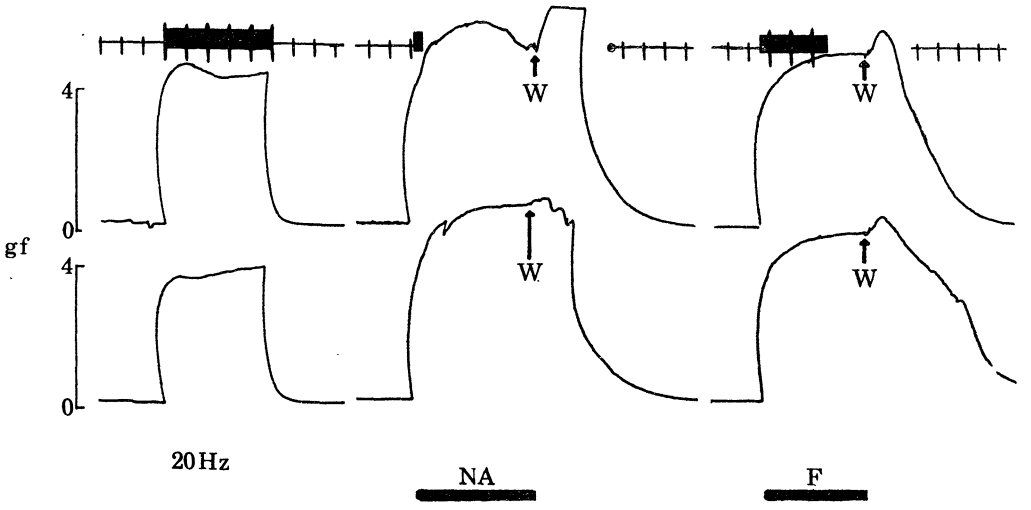

FIGURE 7. Motor responses of a pair of anococcygeus muscles to field stimulation at 20 Hz, 10^{-5} mol/l noradrenaline (NA) and 10^{-5} mol/l furmethide (F). Drugs were washed out at W. The time marker is 1 min. Tension calibration for the middle trace is 2 gf (Gillespie 1972).

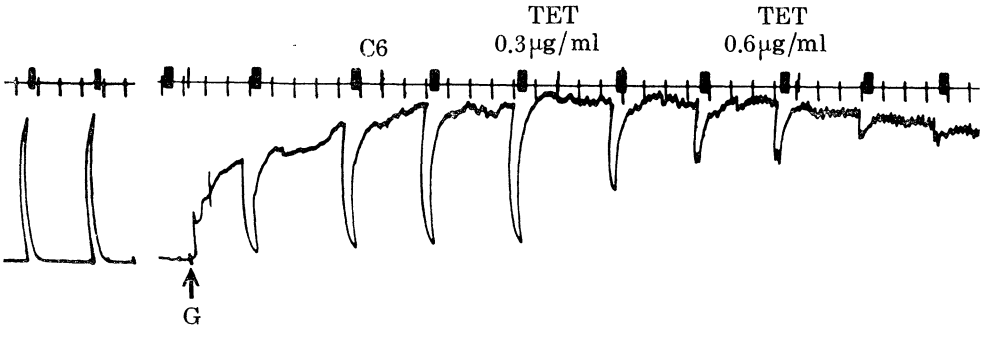

FIGURE 8. Inhibitory responses to field stimulation in the presence of guanethidine (G) 3×10^{-5} mol/l. The response was unaffected by hexamethonium (C6) 10^{-5} mol/l but blocked by tetrodotoxin (TET). Time marker 1 min (Gillespie 1972).

appeared after even fewer pulses. What we believe to be the electrical basis of this phenomenon is shown in figure 10. In the absence of stimulation there is no electrical activity and the membrane is stable at an average of 58.4 ± 4.2 mV. Single pulses at 0.2 Hz caused small depolarizations of a few millivolts but with repetition a second component to the response appeared and quickly attained a considerable size up to 50 mV. All of our evidence suggests that both components are junctional potentials. Spike potentials have never been seen in response to single stimuli. This second component to the response is more easily investigated with short bursts of two to nine stimuli at higher frequency and was shown to be a graded phenomenon. In those

records, where both electrical and mechanical activity was recorded, ring electrodes were used as previously described; where only electrical activity was recorded the partition stimulation method (Abe & Tomita 1968) was used. The pulse duration remained at 1 ms. Figure 11 shows the response of three cells to three trains of three stimuli followed by three trains of six stimuli.

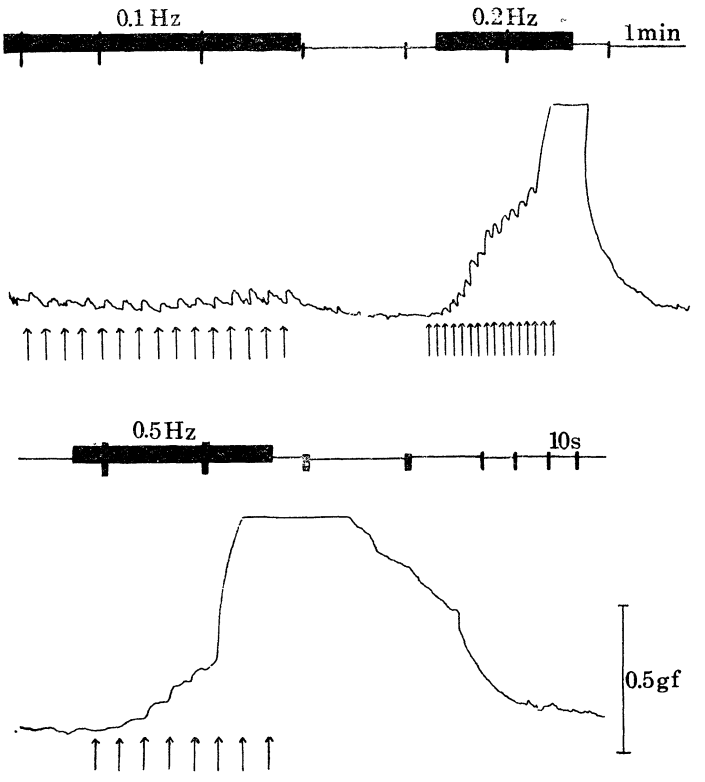

FIGURE 9. The motor responses of the rat anococcygeus muscle to field stimulation during the periods indicated by the bar, showing facilitation and the sudden large increments of tension development. The frequency of stimulation is shown above each record. The recording speed in the lower trace was increased.

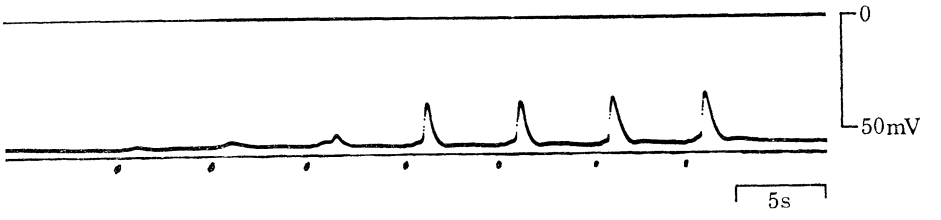

FIGURE 10. Intracellular record of the membrane potential and of facilitation of responses to single pulses, shown by the dots, at a frequency of 0.2 Hz.

In each cell the junctional potential amplitude was graded with the number of pulses and appeared at approximately the same time in all three cells in spite of the varying distance from the stimulating electrode. Figure 12 shows the effects of varying the strength as well as the number of stimuli. The vertical row on the left is the response to nine stimuli and the row on the right to six stimuli. The strength of the stimulation pulses was progressively reduced from the top record to the bottom. In both instances there was a reduction in the size of the response as the voltage was reduced. Figure 13 shows the effect of varying the frequency of stimulation

keeping the number of pulses constant. The frequency is shown on the left. Increasing the frequency again caused a graded increase in the magnitude of the depolarization.

As well as appearing in a graded fashion these responses were abolished in a graded fashion. Phentolamine (2 mg) was added to the 10 ml bath, through which the modified Krebs solution flowed continuously at a rate of about 1 ml/min. Figure 14 shows the effect of phentolamine on the response of a single cell to motor nerve stimulation. The upper panel shows the control response to three periods of stimulation. 1 min after phentolamine was added the two components were still present but were reduced. At 3 min the response was further reduced and both components had disappeared by 10 min.

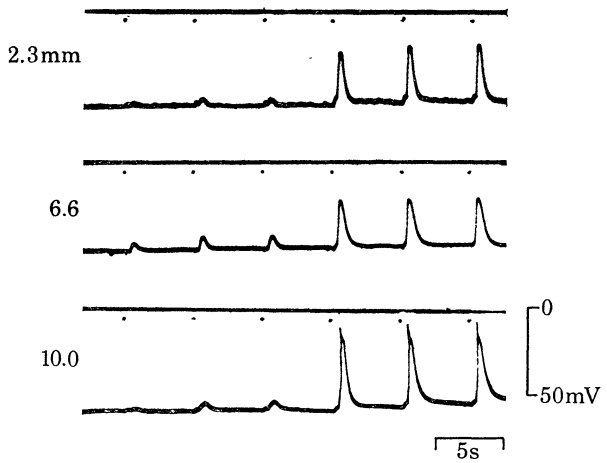

FIGURE 11. Graded responses from cells at the three distances from the nearest stimulating electrode shown on the left of each record. The dots indicate three trains of three stimuli followed by three trains of six stimuli at 30 Hz for each cell. Spike potentials were superimposed on the graded response from the cell at 10 mm.

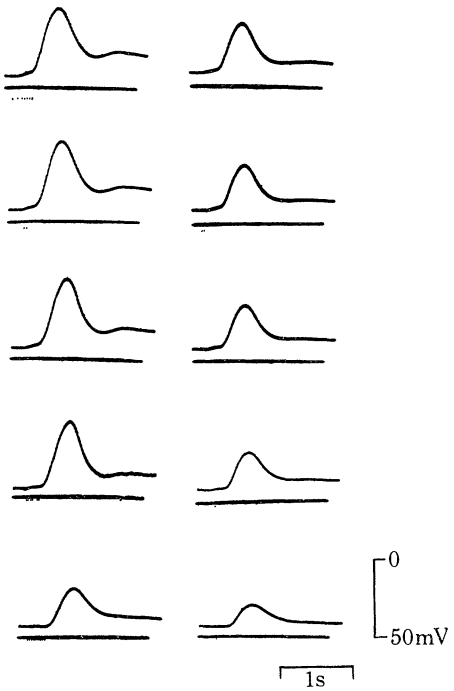

FIGURE 12. The membrane potential response of a single cell to trains of nine stimuli (left-hand column) and six stimuli (right-hand column) at 30 Hz. The strength of stimulation in each column was reduced from top to bottom. The depolarization was graded with the number and strength of stimuli.

In these responses so far there is little or no evidence of spike potentials. If more prolonged trains of field stimulation were given, however, then spikes were occasionally seen. There was no definite threshold but spikes were rarely superimposed on depolarizations of less than 40 mV. In the Krebs solution overshoots were never seen. Spike potentials were more frequently seen in the presence of 1 mmol/l TEA and these often had small overshoots. The repolarization phase was prolonged and the membrane was depolarized to 47.6 mV by TEA.

The effect of more prolonged periods of stimulation is shown in figure 15. Stimulation at 1 Hz caused small depolarizations which culminated in a large depolarization. 3 Hz for a longer period caused large oscillations of potential which outlasted the stimulus duration as did 10 Hz. At 10 Hz one of the large depolarizations had a spike superimposed. When stimulation was

repeated during these membrane oscillations it depressed them as the lower record shows. In another cell a short period of stimulation at 30 Hz caused membrane oscillation; repeating this period of stimulation caused a temporary reduction in the oscillation and finally a prolonged period of stimulation at the same frequency produced a stable depolarized membrane.

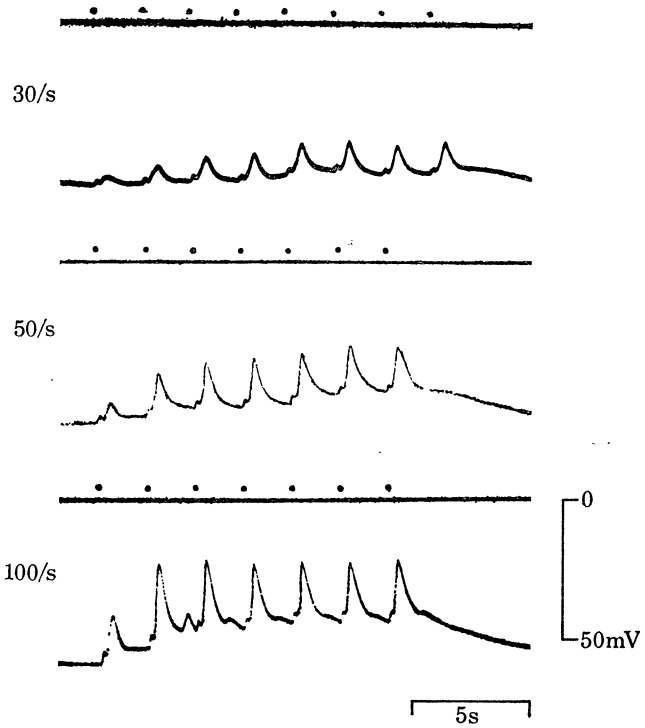

FIGURE 13. The effect of frequency on the membrane potential response of a single cell to field stimulation. Seven or eight short trains of stimuli at 2 s intervals at the frequency shown on the left were given.

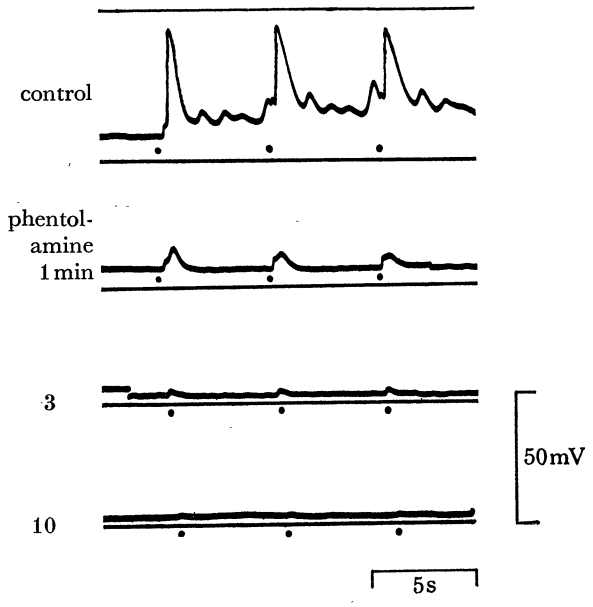

FIGURE 14. The effect of phentolamine (2 mg in a 10 ml bath) on the membrane potential response of a single cell to trains of six stimuli at 30 Hz indicated by the dots. Phentolamine reduced and finally abolished the two components of the excitatory response.

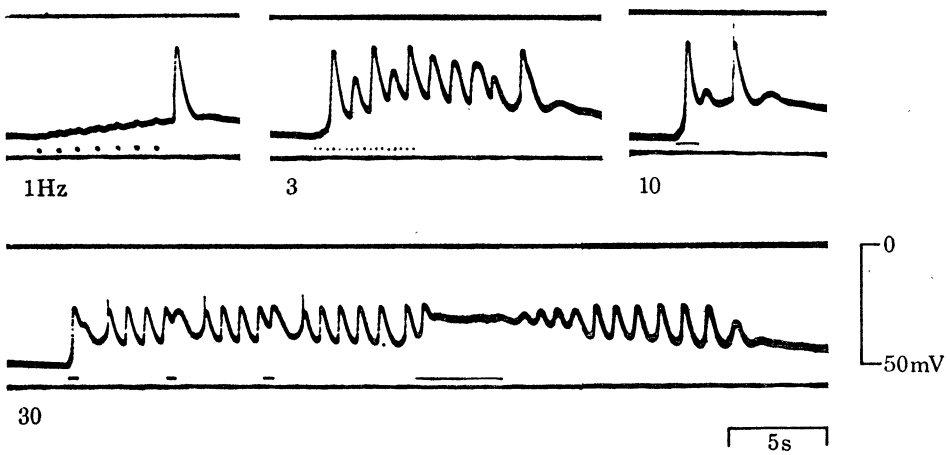

FIGURE 15. Electrical responses to stimulation at 1, 3, 10 and 30 Hz. Each stimulus is marked by a dot. In the upper records activity continued after the end of stimulation. The lower record shows the stabilizing effect of repeated periods of stimulation during this continuing activity.

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A similar pattern of response was seen with drug stimulation (figure 16). These two records show the stimulating effect of noradrenaline and guanethidine added to the bath. The concentrations shown are maximum as the drugs were progressively diluted due to the perfusion through the bath. Both caused depolarization which induced membrane oscillations. These damped out to produce a fairly stable depolarized membrane. The excitability of the membrane was raised by noradrenaline as was seen by comparing the effects of a single nerve stimulus before and after adding the drug. In the early phase of the action of noradrenaline, large depolarizations with superimposed spike potentials were evoked by each stimulus. Spike potentials, however, with both drugs and nerve stimulation were seen in only a minority of the responses.

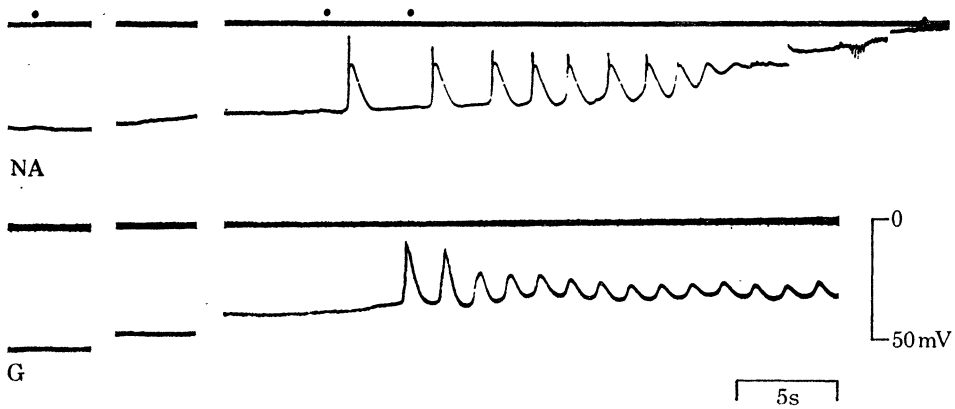

FIGURE 16. Depolarization and oscillation produced by noradrenaline (NA) 10^{-5} g/ml and guanethidine (G) 3×10^{-5} mol/l. Records were taken immediately before and at 40 and 60 s after addition of noradrenaline to the bath and before and at 55 and 115 s after addition of guanethidine. A single stimulus of 1 ms duration evoked a response only in the depolarized muscle.

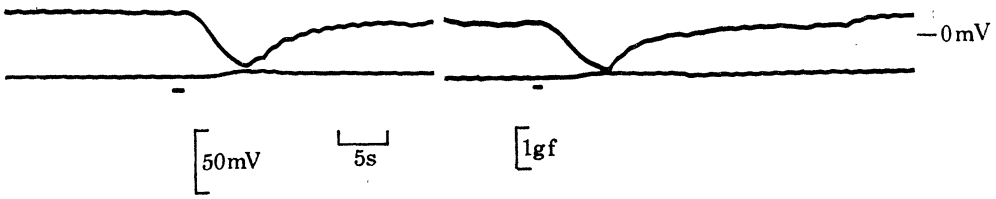

FIGURE 17. Simultaneous recording of electrical (lower) and mechanical (upper) activity from a rat anococcygeus muscle in the presence of guanethidine 10^{-5} mol/l. Field stimulation at 8/s during the period shown by the bar caused mechanical inhibition (downwards). The small oscillations in membrane potential disappeared during and for a short time following stimulation but there was no hyperpolarization. The slight depolarization is probably a mechanical artefact.

The response to stimulation of the inhibitory nerves is shown in figure 17. The electrical response was recorded from a single cell with a microelectrode while the mechanical response represents the whole muscle. Guanethidine had been added to the perfusion fluid with the result that the membrane was depolarized to 25 mV. Superimposed on this was a fine oscillation at about 1/s and the muscle was in maintained contracture. Field stimulation caused mechanical relaxation. The oscillations of the membrane potential were abolished during, and for a short time after the stimulus, but in spite of the depolarized state of the muscle there was no evidence of hyperpolarization. The small depolarization which occurred here during the period of stimulation was not a consistent observation and we believe it was a mechanical

artefact. The abolition of the small oscillation in membrane potential by field stimulation may indicate an increase in membrane stability.

The inhibitory effect was graded with frequency. Figure 18 shows the effect of 10 s periods of stimulation at various frequencies. The maximum mechanical inhibition was at 4/s but again at no frequency was there any consistent evidence of hyperpolarization. Figure 19 shows the effects of varying the number of pulses by opening the stimulator gate for various times; stimulation therefore lasted for the period shown by the black bar. Mechanical inhibition increased progressively with the number of pulses but again the only change in membrane potential, which can be seen best in the second record, was the disappearance of the membrane oscillations during and for a short period after the stimulus.

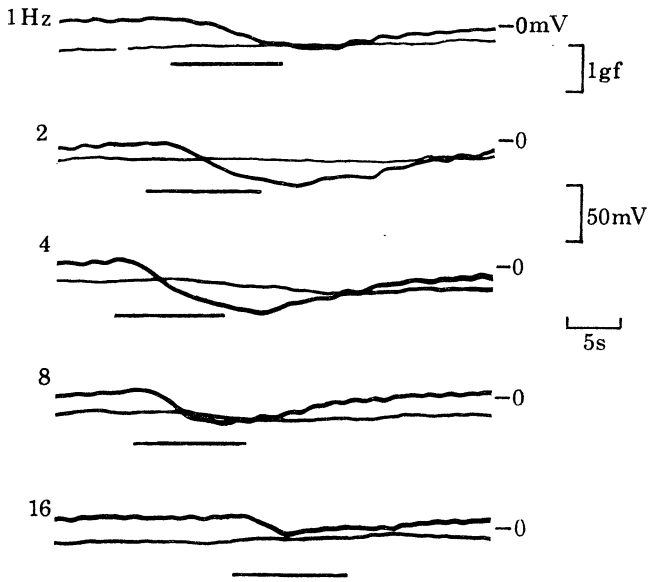

FIGURE 18. The effect of frequency of stimulation from 1 to 16 Hz on the response of the rat anococcygeus muscle in the presence of guanethidine 10^{-5} mol/l. The upper trace is the mechanical response of the whole tissue with inhibition downwards and the lower trace is the electrical response of a single cell. The mechanical inhibition is graded with frequency with a maximum at 4 Hz.

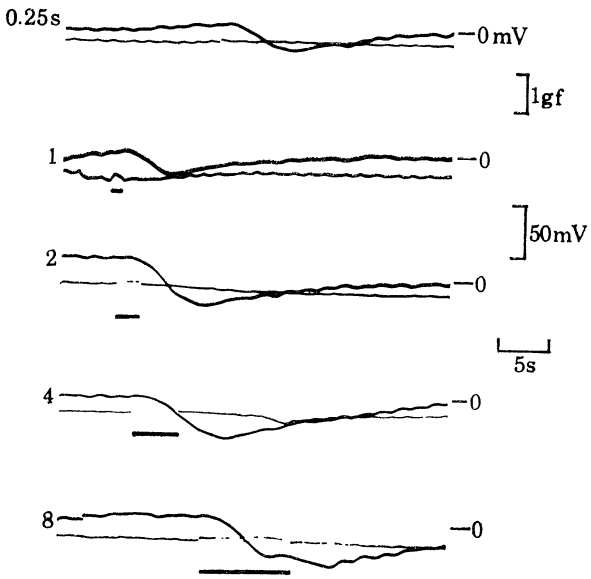

FIGURE 19. The effect of increasing the duration of trains of stimuli at 16 Hz from 0.25 to 8 s on the mechanical (upper) and electrical activity of the rat anococcygeus muscle in the presence of guanethidine 10^{-5} mol/l. During stimulation, especially in the upper two records, the small oscillations in membrane potential were abolished; there was no hyperpolarization.

In summary then the anococcygeus is a muscle with a stable high membrane potential and no spontaneous activity. Excitation by the motor nerve appears to be due to graded junctional depolarizations rather than spike potentials and inhibition to membrane stabilization without hyperpolarization although the depolarized membrane might be expected to be ideally suited to displaying hyperpolarization.

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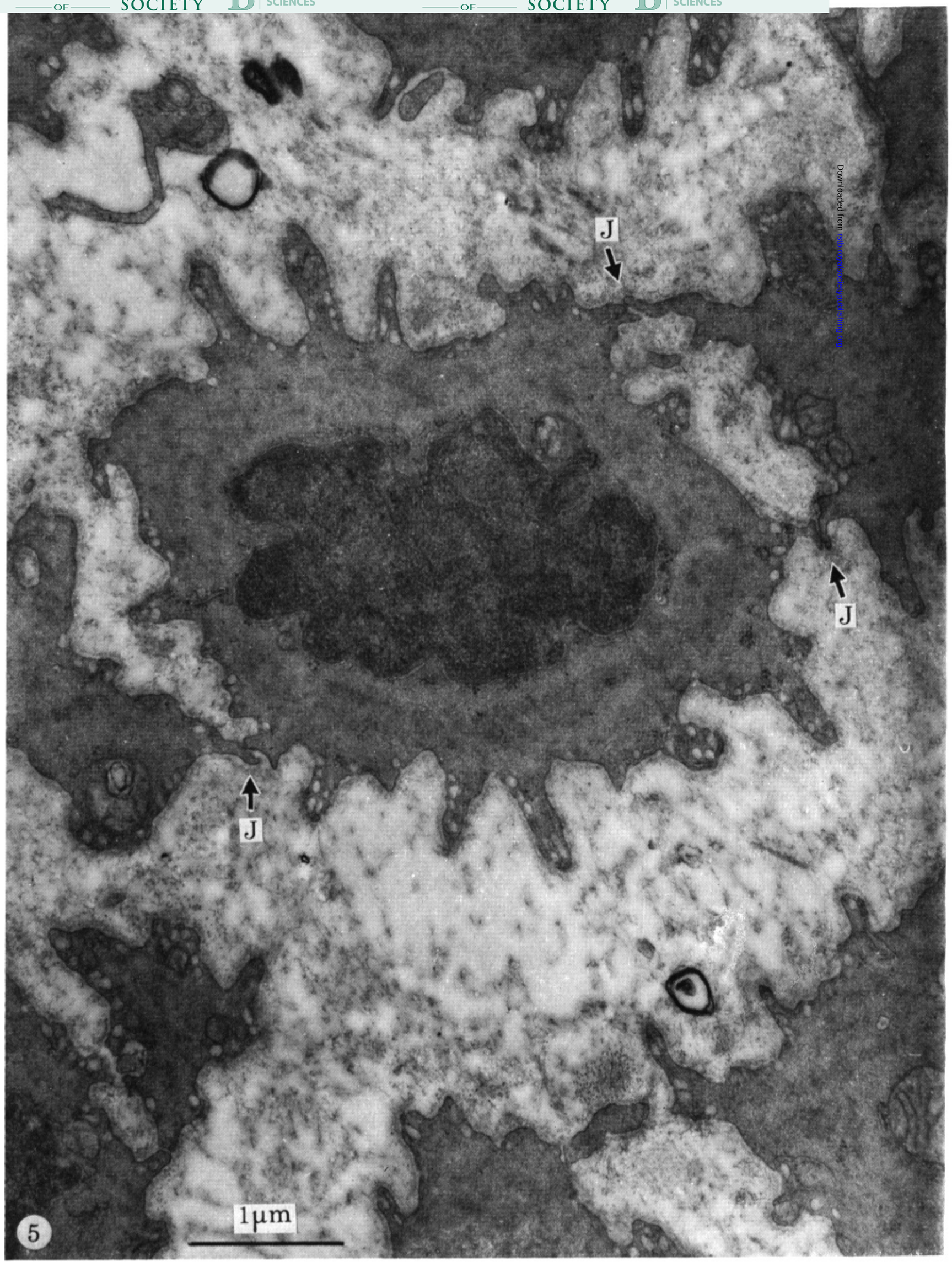

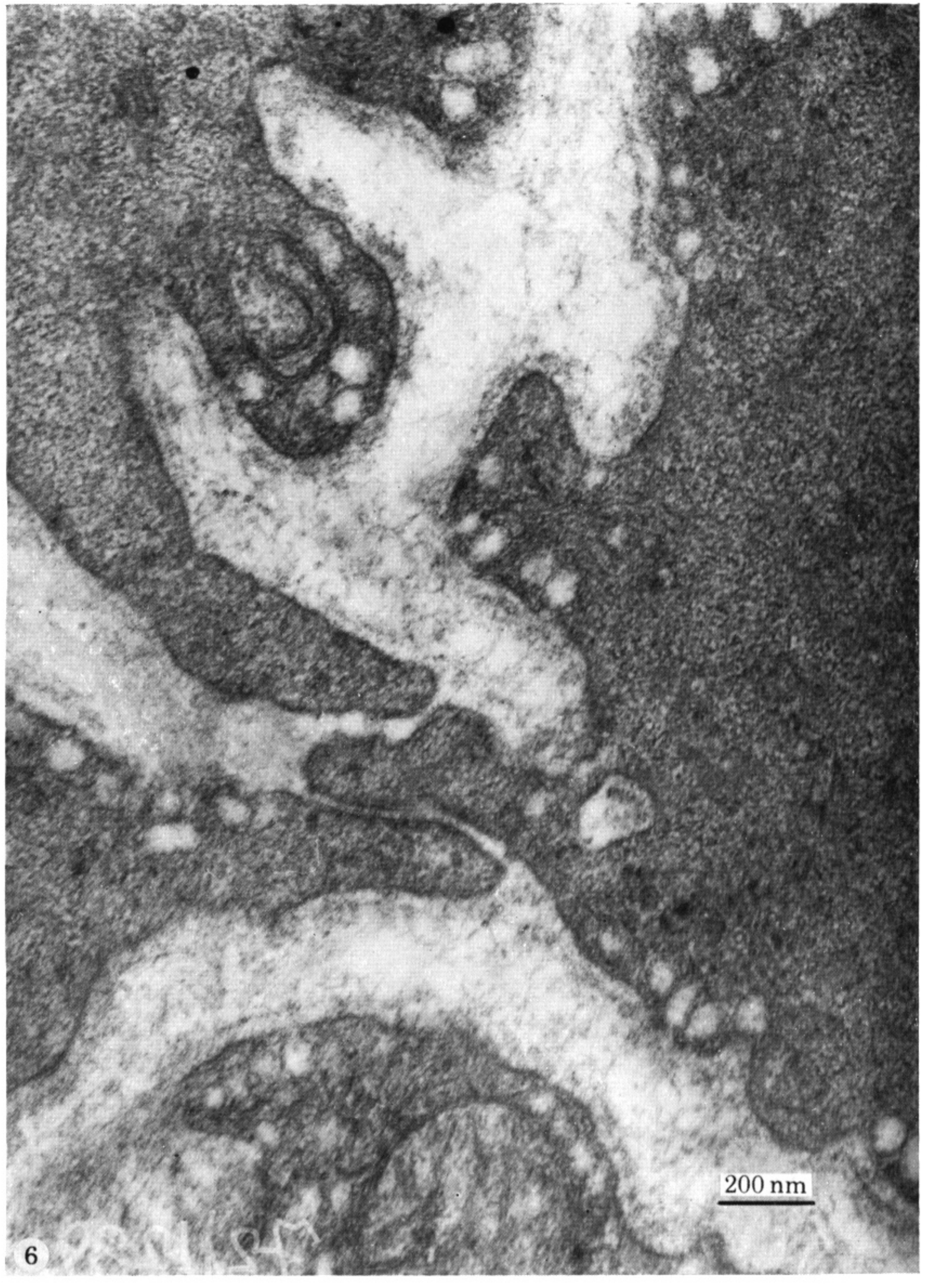

FIGURE 5. Transverse section of the anococcygeus muscle showing a single smooth muscle cell sectioned at the level of the nucleus, with several neighbouring cells. Caveolae, surface projections and close junctions (J) with two other cells are visible.

FIGURE 6. Close junctions between three neighbouring cells, with a minimum separation of 14 nm. Basement membrane is present in the gap.