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MEMBRANE POTENTIALS OF SMOOTH MUSCLE FIBRES IN THE RABBIT'S SPHINCTER PUPILLAE

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The measurement of mammalian smooth muscle potentials using intracellular microelectrodes of the type first described by Ling & Gerard (1949) for striated muscle presents several difficulties. First, the smooth muscle cell is smaller than the striated muscle fibre and thus the danger of damage during the puncture is so much greater. Secondly, in most organs containing smooth muscle this is surrounded by connective tissue too hard to penetrate with a fine glass electrode. In softer organs such as the intestinal tract, where there is less connective tissue interposed between muscle fibres, a third difficulty is presented by their spontaneous activity. During the muscle movements the electrode may slip and any observed change in potential may be an artifact due to a changed position of the electrode in relation to the cell, indistinguishable from true potential changes related to the cell's activity. Besides, it might be difficult to determine a 'resting potential' in muscles with rhythmic activity which are never truly at rest.

For these reasons a tissue has been chosen which does not exhibit spontaneous activity, which can be obtained in a sufficiently thin layer to be adequately oxygenated in an isolated organ bath and well illuminated for the electrode to be inserted under microscopic observation. The iris of albino rabbits proved to be suitable because the absence of pigmented epithelium allows distinction of the component parts, especially of the sphincter pupillae. This preparation has therefore been used to collect information on the resting potential in at least one type of mammalian smooth muscle.

METHODS

Albino rabbits were killed and bled out. The eyeball was removed. By cutting the sclera along the edge of the cornea, the lens, iris and cornea were detached. The lens and iris were isolated together. When such preparations were used and attempts were made to insert a microelectrode into the iris, breakage was frequent because a thick layer of connective tissue anterior to the sphincter pupillae had to be penetrated. For this reason the iris was inverted and a preparation was

obtained with the muscle component nearer the surface. The lens was carefully separated from the iris and placed in a shallow Perspex cup as shown in Fig. 1A. The iris was then replaced on top of the lens inverted so that the posterior surface lay uppermost (Fig. 1B). It was held in position by a Perspex ring with a sleeve which fitted the cup (Fig. 1C). When this was slid on from the top some of the outer edge of the tissue was caught with it and thus the iris was fixed on top of the lens (Fig. 1D). In some experiments two Perspex rings were used, one fitting into the other and the inner ring fitting the cup. The lens was placed in the cup and held by the first ring; then the iris was placed on top and held by the second ring. The preparation in its Perspex holder was transferred to the constant temperature bath. The outer bath was filled with water at 39° C con-

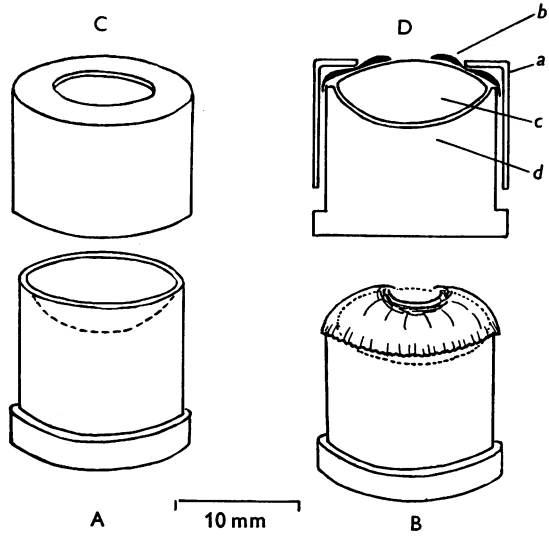

Fig. 1. Method for holding the rabbit's iris. A, Perspex cup for the lens; B, the lens (dotted line) is placed in the cup and the iris spread over it; C, Perspex ring; D, cross-section of mounted preparation. (*a*) Perspex ring, (*b*) iris, (*c*) lens, (*d*) Perspex cup.

stantly circulated from a thermostatically controlled heating bath. The inner bath contained a modified Krebs solution of the following composition. To 1 l. distilled H_2O was added 7·8 g NaCl, 0·35 g KCl, 0·165 g NaH_2PO_4, and 1·37 g $NaHCO_3$. When this solution had been saturated at 37° C with a gas mixture of 95% O_2 and 5% CO_2, 1·4 ml. 20% $CaCl_2$ solution, 1·0 ml. 1% $MgCl_2$ solution and 1·4 g dextrose were added. It flowed from a reservoir continuously at a rate of 2 ml./min through two coils of polythene tubing lying in the outer bath before entering the inner bath, whose fluid level was kept constant by suction. The inner bath contained also the indifferent electrode and a thermometer. Another thermometer was inserted in the outer bath. As the inner bath was only warmed at the sides but not from underneath its temperature did not reach that of the outside bath but remained at 35° C.

In the early part of the investigation the electrode was supported on a brass guide which could be advanced by means of micrometer head (accuracy 5–10 μ), and the other necessary movements were obtained with a Palmer adjustable stand and a microscope mechanical stage. In the later experiments we used an oil-operated micromanipulator (Matthews, 1952, accuracy 1–2 μ), on an anti-vibration mounting.

Substage illumination was provided from a projector lamp, microscope mirror and condenser. Manipulations were observed through a Zeiss microscope with a long-distance objective and

a magnification ×80, and in later experiments through a Zeiss Opton binocular dissecting microscope with a range of magnification from ×6 to ×160.

Microelectrodes were made from Pyrex or Monax tubing as described by Ling & Gerard (1949), with the fine end drawn out as short as possible. A stubby, non-flexible type with an even tapering towards the tip was found to give the best results. All electrodes were inspected with a water-immersion objective and only those with a tip of less than 0·5 μ outside diameter were used. They were filled with 3 M-KCl.

The pre-amplifier had a balanced input of electrometer acorn pentodes type 954, similar to that suggested by Bishop (1949). After one stage of amplification the output was converted to a single-sided signal and fed into a commercial d.c. oscilloscope (Cossor type 1049) via a cathode follower. The input grid current was not greater than 10^{-11} A and the time constant was less than 200 μsec with a source impedance of 50 MΩ. A calibration signal was obtained from a potentiometer checked with a standard cell (Nastuk & Hodgkin, 1950). The resistance of the electrodes varied between 17 and 28 MΩ, the average being 20 MΩ. The grid current flowed towards the grid and would give a negative potential if the resistance of the electrode tip was increased by the material through which it passed. By calculation a 20 MΩ increase in resistance would lead to 0·2 mV increase in negative potential.

RESULTS

Measurements of muscle fibres

When the iris preparation was observed with a microscope and a strong substage illumination, circular bundles of fibres were clearly seen close to the edge of the pupil; farther away, towards the periphery, radial fibres were seen. Both layers overlapped, but close to the pupil the circular fibres were near the surface. It was not possible to distinguish with certainty connective tissue fibres from muscle fibres, both being closely interwoven.

When, after treatment with concentrated KOH, the inner rim of the iris was teased and the tissue components were inspected with a microscope and high magnification, many connective tissue fibres were seen and only comparatively few spindle-shaped smooth muscle cells. They measured 3–10 μ in diameter and 10–30 μ in length. The procedure of separating them by treatment with KOH probably led to shrinkage. Measurement of cross-sections of single fibres in histological slides gave a diameter of 5–6 μ. The figure for shrinkage which is usually given is that fixation produces an average reduction to 50% of the original size. Thus a diameter of little more than 10 μ would be expected *in vivo*. In the living preparation it has been impossible to discern spindle-shaped cells; they always appeared to be bundles of uncertain definition.

Electrical potential changes

When the microelectrode was inserted into the tissue it was possible with a microscope to observe the approach of the electrode towards a muscle bundle, but it was impossible to see the actual tip entering a muscle cell. Dimpling of the tissue nearer the surface occurred when the electrode was advanced more deeply. If dimpling occurred on touching the tissue it was a certain sign of a blunt tip and no measurement of electrical potential could be

obtained. With electrodes which did not produce any visible dimpling on contact with the iris potentials were recorded as shown in Fig. 2. At the first contact with the tissue a change of potential from 0 to 2–5 mV was usually observed, then, near the edge of the pupil, potentials of about 60 mV negative to the outside solution could be recorded at a depth of about 10 μ from the surface. These were only rarely obtained instantaneously and if so most likely near the edge of the pupil. The change did not occur as abruptly as we observed

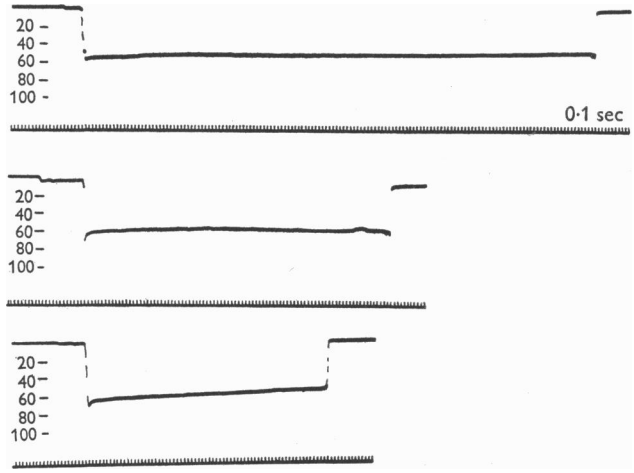

Fig. 2. Membrane potentials recorded from the sphincter pupillae close to the edge of the pupil.

it to occur in frog sartorius fibres. In the iris a potential between 20 and 40 mV. was usually obtained before an abrupt change to 60 mV or more occurred. Near the edge of the pupil the final value was obtained more or less instantaneously as shown in Fig. 2. But by far the largest number of records showed a stepwise entry and these steps were the more pronounced the further the site of puncture was moved towards the periphery.

Fig. 3 shows examples recorded at 200–300 μ distance from the pupillar edge. In all three records potentials of about 60 mV were recorded near the surface and, as the electrode was advanced into the tissue, the potential fell again; however, it did not return to the base-line. An intermediate level between 20 and 40 mV was maintained until, as the electrode was moved up to about 50 μ into the tissue, one or more changes to 60 mV or above occurred. When the final value was attained it was often possible to move the electrode over 20 μ or more without appreciably altering the potential. On withdrawal of the electrode the potential first returned to an intermediate level not to zero. Often a rise, corresponding to the high potential recorded near the surface on entering the tissue, occurred before the return to the base-line as the electrode came out.

In Fig. 4 are shown examples of records obtained at a distance of 500–600 μ peripheral from the edge of the pupil. Again the intermediate levels were maintained while the electrode was lowered to a depth up to about 100 μ. In this region of the iris potentials of 60 mV were rarely seen near the surface,

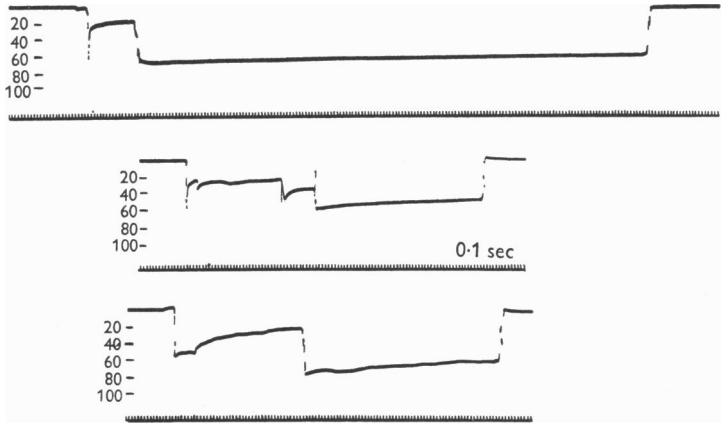

Fig. 3. Potentials recorded when the electrode was inserted into the iris at a distance of 200–300 μ peripheral from the pupil and advancing it about 50 μ.

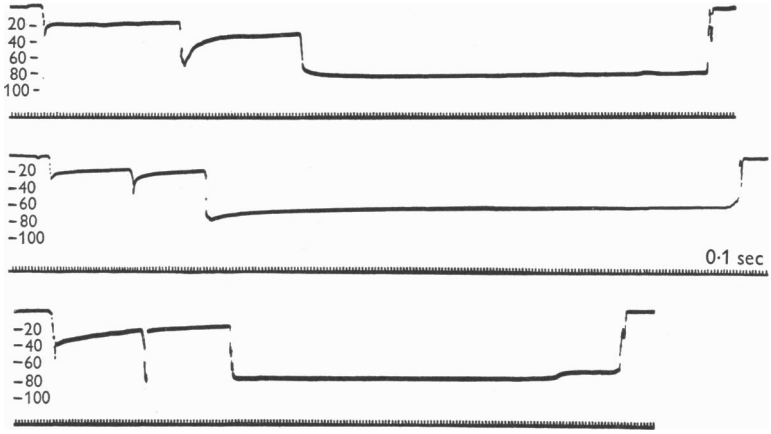

Fig. 4. Potentials recorded on inserting the electrode at a distance of 500–600 μ peripheral from the edge of the pupil and advancing it up to 100 μ.

but usually after advancing the electrode for a considerable distance into the iris tissue. How far, however, the electrode actually advanced into the tissue and how far it pushed the tissue with it could not be ascertained. The latter possibility is likely as dimpling of the surface was always noticed when the tip was pushed deeply inside the tissue.

Correlation of anatomical distribution of tissue components with electrical potential levels within the iris

An anatomical explanation of the potential changes recorded may be found on inspection of Fig. 5. This represents a drawing of a serial section of a rabbit's iris, traced from a projection of the histological preparation. It was the central section and points 100 μ apart have been marked at radially

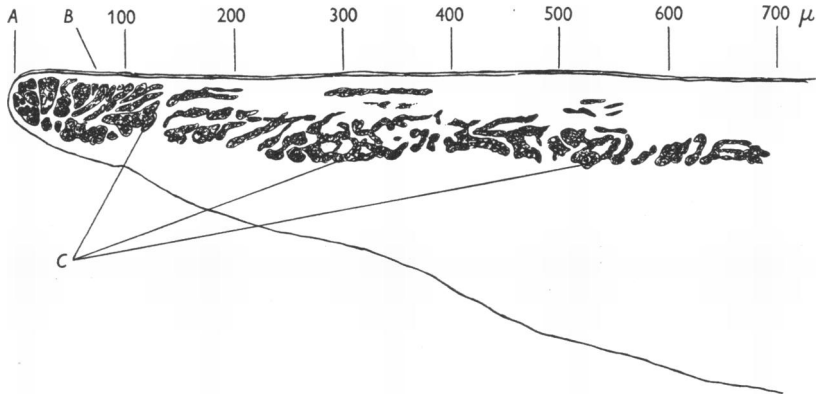

Fig. 5. Histological preparation of rabbit's iris. Tracing from projected central section. *A*, edge of pupil; *B*, epithelial layer of posterior surface lying uppermost; *C*, sphincter pupillae in cross-section. Note large areas of connective tissue between bundles of muscle fibres.

increasing distances from the pupil where a microelectrode might have been inserted. The drawing shows that near the pupil the smooth muscle lies closest to the surface and that towards the periphery it can be reached only by penetrating more deeply through the connective tissue. In the iris (as distinct, for example, from a frog sartorius preparation) the electrode had to pass through several kinds of tissue. It first penetrated an epithelial layer, and then, alternately, spaces of connective tissue and of smooth muscle. Thus results such as those shown in Figs. 2–4 might be expected. When the final value was reached the potential was fairly well maintained, as is shown in the records. A very gradual decay was nearly always seen, but many potentials were maintained at no less than 90% of the original for as long as 5 min.

Interpretation of results and frequency distribution of muscle potentials

Several experiments were carried out to ascertain how far the records could be interpreted as potentials arising from muscle cells. First, potentials of the order of 60 mV could be obtained only in that part of the iris where circular muscle fibres could be seen, an area which agreed with that to which, in histological preparations, the sphincter pupillae is confined. Outside this region

potentials of 30 mV, sometimes even 40 mV were recorded; the change of potential, however, was not abrupt but gradual.

Treatment of an iris preparation with 0·01 M-KCN lowered the potential only by 10%, but after exposure to 10% chloroform no more than 15 mV could be obtained at any place in the tissue. Another iris preparation, in which potentials of 52–60 mV were easily obtainable, was heated to 55° C for several minutes. It was then cooled again to 35° and, on sampling several places, potentials up to 20 mV were still recorded but none higher than that. The iris was then removed and replaced by the iris from the other eye of the same rabbit. The same electrode was used and potentials of 45–65 mV were again recorded. This preparation was now exposed for several minutes to 50% ethyl alcohol. After replacing the alcohol and careful washing with the Krebs solution, measurements were made and the highest potential obtainable was 25 mV at a depth of 80 μ inside the preparation.

Finally, while a potential of 65 mV was steadily maintained in a fibre, KCl was added to the bath to produce 30 times the normal concentration. The potential fell immediately to 10 mV. This might have been due to movement as the pupil constricted, but on sampling several places no more than 15 mV could be obtained anywhere.

The activation of fibres by smaller doses of KCl or by acetylcholine always led to a slow fall in potential. However, movement artifacts could not be excluded, as the preparation was not fixed isometrically. Experiments on active tissue were therefore left to further investigation.

With the data available from the foregoing experiments it seemed justifiable to exclude potentials below 25 mV as not originating from muscle fibres. Potentials up to 40 mV might be recorded from damaged muscle cells. On the other hand, they might arise from different cells such as epithelium or connective tissue. It was not possible to decide this with certainty. However, regions of lower potential were found, in agreement with the anatomical distribution of connective tissue between bundles of muscle fibres, as the electrode was gradually advanced into the iris tissue. These intermediate readings, either preceding regions of higher potentials or interposed between such regions, were therefore discarded. The remaining lower records were included in the selection of fibres which was made according to criteria similar to those applied by Nastuk & Hodgkin (1950) to frog sartorius fibres and more recently by Kuffler & Vaughan Williams (1953) to the slow skeletal muscle fibres in the frog. They were (*a*) a sudden entry, and (*b*) maintenance of the potential level. In one series, obtained with the micrometer screw manipulator, the average potential of 186 impalements was found to be 62·9 mV (S.D. $\pm$ 9 mV). In a second series, obtained with Matthews's oil-operated micromanipulator, the mean figure for 174 fibres was 57·6 mV (S.D. $\pm$ 11 mV). The frequency distribution for the total of 360 observations is represented in

Fig. 6. Though the peak frequency is at 60 mV the true value may be higher, as there is almost a plateau between 60 and 70 mV.

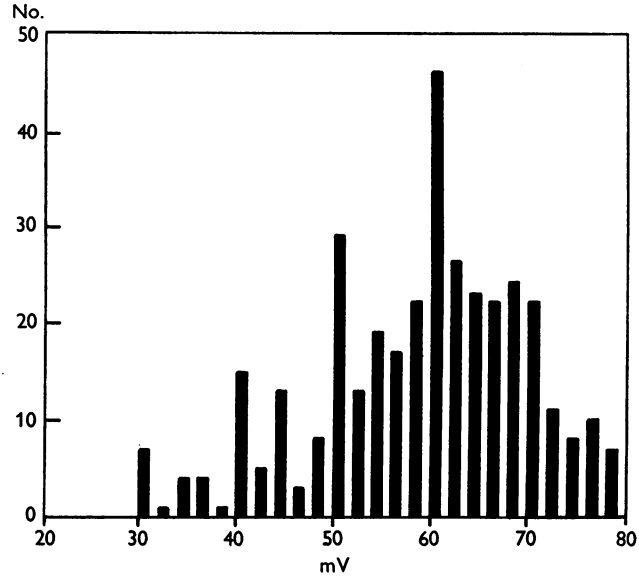

Fig. 6. Frequency distribution of 360 potentials recorded in the sphincter pupillae of the rabbit's iris.

DISCUSSION

The sphincter pupillae was chosen for the determination of the normal membrane potential in smooth muscle fibres because it does not exhibit any spontaneous movements. The mean value of the resting potential was 60 mV. Exploration of the iris with microelectrodes revealed regions of low potentials (20–40 mV) alternating with regions of higher potentials (60 mV). The distribution of these different potential levels agreed well with the distribution of connective tissue and muscle fibres found in histological sections. It may be that many of the potentials recorded at the surface of the iris arose from the epithelial layer and dilatator pupillae which is a myoepithelium. Hickman & Woodbury (1951) have reported membrane potentials of 30 mV from the epithelium in various parts of the frog's intestinal tract. But below the surface the main constituent of the iris is connective tissue, and it was surprising to find that electrical potentials between 20 and 40 mV could be recorded from these regions, and also in parts of the iris which contained no muscle. One might suspect them to be mechanical artifacts produced by bending of the electrode or occlusion of the tip against an impenetrable resistance. The latter was believed to be the reason for occasional very high

potentials recorded if the electrode was advanced too deeply into the tissue and possibly getting close to the underlying lens. These potentials were, however, unstable and could be produced by deliberately pressing the electrode against the lens. A record of a potential of 87 mV reported in our previous communication (Bülbring & Hooton, 1953) could not be repeated when using Matthews's oil-operated micromanipulator with a more controlled movement. 78 mV was the highest potential which was repeatedly recorded. When a microelectrode was inserted into a 6% agar block made with Ringer solution containing the same salt concentrations as that in which it was immersed, no change of potential was observed on pushing the electrode from the solution into the solid agar. However, within connective tissue of similar solidity potentials of some magnitude were observed, though they were never as high as those arising from the muscle.

Considering the potentials arising from the muscular tissue several questions remained. Were these potentials recorded from single cells and were they the true potentials or those of damaged cells? The observation that the electrode tip could be moved without considerable change in potential over a greater distance than the diameter of a single cell could be explained by the possibility that, on lowering the electrode, the gradually thickening shaft might push the tissue with it, as indicated by the dimpling on the surface. The tip would then remain inside the same cell which was moved down in front of the electrode. In this process the potential might remain constant, or, if the cell was damaged and leakage occurred, it might fall to lower values until the electrode penetrated a neighbouring cell. The interpretation of the potentials recorded from the muscular tissue was further complicated by the potentials apparently arising from connective tissue spaces. In many instances more or less steady potential levels, which never fell to the base-line, were recorded while the electrode was inside the tissue. If these potentials continued right up to the surface of the muscle cells the membrane potential of the muscle would not be 60 or 70 mV but the difference between this and the steady level recorded before the electrode tip entered the fibre. However, the regularity with which potentials of the order of 60 mV were recorded near the edge of the pupil, where the muscle was close to the surface, made it likely that the true value was in this region.

The iris preparation was not found suitable for the study of activity changes because movement artifacts could not be excluded.

SUMMARY

1. The resting potential of smooth muscle fibres has been measured, using intracellular electrodes, in the sphincter pupillae in the isolated iris of albino rabbits.

2. The average potential was 60 mV.

3. The potential was often maintained with no more than 10% decay for several minutes.

4. The possibility is discussed that lower potentials, in the region of 40 mV, originate from the epithelium, myoepithelium, and possibly from connective tissue cells.

We are very grateful to Prof. B. Katz and to Prof. A. L. Hodgkin for much valuable advice, and to Prof. Sir Bryan Matthews for allowing his oil-operated micromanipulator to be copied. The collaboration of Mr O. B. Saxby in constructing this and most of the other apparatus was invaluable. One of us (E.B.) would like to express her thanks to the Government Grant Committee of the Royal Society for a grant covering the main cost of the equipment needed for this work.

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