Brit. J. Pharmacol. (1963), **21**, 331–338.

THE RESPONSE OF THE ISOLATED SKIN OF RATS TO DRUGS AND ELECTRICAL STIMULATION

BY

K. HELLMANN

From the Chemotherapy Unit, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C.2

(*Received June* 21, 1963)

A new method of transmural stimulation (Hellmann, 1963b) has been used to examine the responses of the rat panniculus carnosus, a skeletal muscle which lines the skin. This preparation responded to drugs and to electrical excitation in the same way as other mammalian skeletal muscle preparations. The relaxation phase, when examined with fast recording systems, was complicated by what appeared to be the " tone " of the muscle.

When pilomotor muscles are present in isolated skin preparations, physiological competence can be gauged by the continued ability of these muscles to respond to stimulation ; thus, in cat isolated skin this activity remains unimpaired for at least 3 days (Hellmann, 1963a). Tubes of skin from cats' tails were used and the pilomotor muscles stimulated transmurally by means of coaxial electrodes but, by using a new method of transmural stimulation which permits small thin discs of tissue to be used (Hellmann, 1963b), the survival of responses from the muscle elements of isolated skin from almost any site may be examined. In this way it is possible to study the survival of responses both of the pilomotor muscles in rat skin and of the panniculus carnosus which lines it.

The panniculus carnosus is a sheet of voluntary, striated muscle under the skin of most mammals, particularly those in the more primitive species, and its fibres insert directly into the skin. Remnants of the muscle are present in man, where it is represented by the facial musculature and the platysma (Clark, 1958). This simple muscle seems never to have been used for pharmacological or physiological experiments, though its accessibility, wide distribution and robust character make it very suitable for this kind of work, and because it is a wide, but thin, muscle sheet it would seem to be particularly valuable for *in vitro* studies. Before testing the preparation over a period of days, it was necessary to define its acute reactions to drugs and electrical stimulation *in vitro*. The present paper describes this work, a preliminary account of which has already been given (Hellmann, 1963b).

METHODS

Rats were briefly anaesthetized with ether to allow an intraperitoneal injection of urethane (125 mg/100 g body weight). In some instances urethane was administered without ether. The back of the rat was shaved and an area of skin, extending approximately 3 cm laterally on both

sides of the midline and 10 cm from the base of the tail towards the head, was marked out and quickly dissected off. The skin of the back of the rat only loosely adheres to the underlying tissues and large areas can be removed rapidly without difficulty. The panniculus carnosus is firmly attached to the skin and is removed with it. It is well developed in the rat but varies somewhat in thickness. In the midline of the dorsal region it is approximately 0.25 mm thick and reasonably uniform, and it becomes somewhat thicker as it reaches ventrally.

The isolated piece of skin was laid epidermis downward and the panniculus carnosus was kept moist with cold Krebs solution. Loose fascia was quickly trimmed off and a disc of skin 1 in. in diameter was cut out with a punch. The disc was rinsed in Krebs solution and then clamped between the two Perspex rings of a bath devised for transmural stimulation of non-tubular tissues (Hellmann, 1963b; Hellmann, Stedman & Duke, 1963). This bath was designed to accommodate and hold rigidly the Perspex rings with the tissue clamped between them. Coiled platinum wire electrodes, of 0.022 in. diameter and placed in the Krebs solution above and below the tissue, enable it to be stimulated transmurally. Positive pressure was exerted by Krebs solution in a reservoir, connected by a tube to the Krebs solution under the tissue. This pressure causes the tissue to balloon upwards. Stimulation shortens the muscle fibres which moves the tissue downwards. The subsequent increase in pressure in the lower compartment is detected by a pressure transducer, the output of which is amplified and recorded on pen or ultra-violet recorders. The pressure changes have also been registered by feeding the output from the amplifier to a dual-channel oscilloscope (Solartron). Contractions are either isotonic or isometric, depending on whether or not the connexion to the reservoir is clamped off. In all experiments referred to in this paper contractions were isometric.

The ring-tissue assembly was placed in the bath with the panniculus carnosus facing upward and the epidermis downward. The Krebs solution in the upper compartment was bubbled constantly with a mixture of 95% oxygen and 5% carbon dioxide, whilst more Krebs solution was allowed to flow through the upper compartment at controlled rates from a reservoir. The volume of the upper compartment was approximately 10 ml. All experiments were carried out at room temperature (20° C) in an air-conditioned room, this ensuring that the apparatus and Krebs solution were at the same temperature and so reducing fluctuations of output from the temperature-sensitive transducer. Contractions of the muscle at 20° C were greater than at higher temperatures and relaxations were not noticeably longer.

In one experiment the panniculus carnosus was detached from the disc of rat skin with scissors and the skin was then set up as before.

The skin preparations were stimulated with rectangular-wave shocks of 0.04 msec duration, unless otherwise indicated. Supramaximal shocks (60 V) were used at all times. The frequency of stimulation was 0.45 shocks/sec, unless otherwise stated.

Drugs used were: acetylcholine perchlorate; (−)-adrenaline bitartrate; decamethonium iodide; neostigmine sulphate; phenoxybenzamine hydrochloride; potassium chloride; and (+)-tubocurarine chloride. The weights of drugs refer to the salts and concentrations are expressed as final concentrations in g/ml. The Krebs solution had the following composition in g/l.: NaCl 6.9; KCl 0.35; KH_2PO_4 0.16; $CaCl_2\,2H_2O$ 0.37; $MgSO_4\,7H_2O$ 0.29; glucose 1.0; and $NaHCO_3$ 2.1. It was bubbled constantly with a mixture of 95% oxygen and 5% carbon dioxide.

Some preparations were fixed in formol-saline, embedded in paraffin, and cut into 5 μ sections which were stained with haematoxylin and eosin.

RESULTS

General

The contraction phase. The rat skin preparation showed no spontaneous activity. Responses to continuous stimulation with pulses of the same voltage, duration and frequency remained constant for hours. The size of the contractions varied with the frequency and voltage of stimulation and with the drugs added to the bath. Small

variations in temperature had little effect on the size of contractions, but the response varied with changes in the load applied to the tissue. Maximal contractions could be obtained with quite small loads (50 to 100 mm of Krebs solution). When pressures were increased or decreased above or below the optimum pressure, contractions became smaller. When contraction heights were calibrated with a mercury manometer, it was found that a single maximal contraction exerted a pressure equal to 10 mm Hg.

The relaxation phase. Responses were usually recorded on pen recorders and contractions then appeared to be mirror images of relaxations (Fig. 1), but recordings made with an ultra-violet light recorder (Fig. 2) and confirmed by examina-

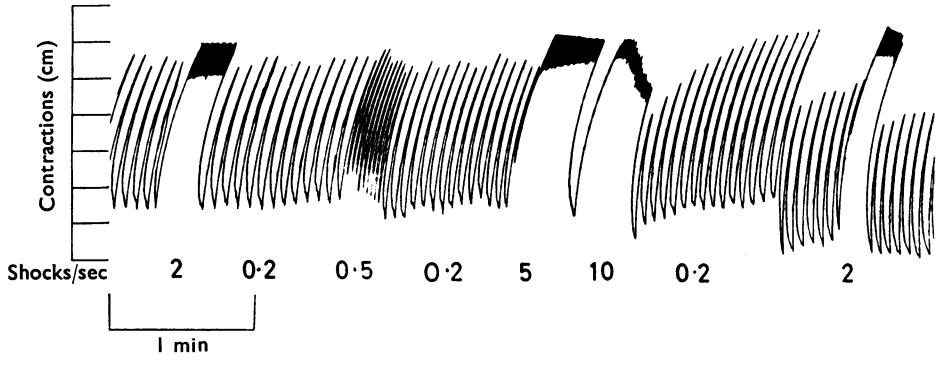

Fig. 1. Contractions of a rat panniculus carnosus preparation registered with a pen recorder. The response scale is recorder deflection in cm. Responses at 20° C to different frequencies (values below trace) of supramaximal stimuli. The shock duration was 0.04 msec.

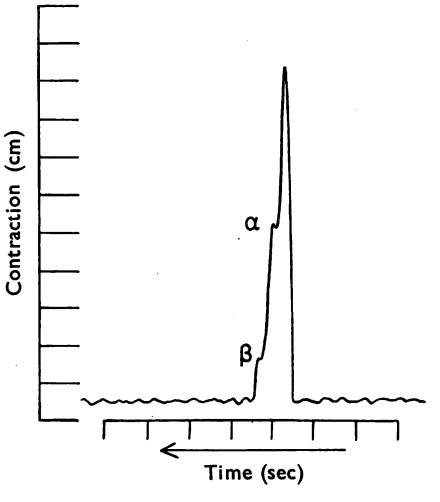

Fig. 2. Contraction of a rat panniculus carnosus preparation registered with an ultra-violet light recorder. Ordinate scale as in Fig. 1. The record reads from right to left, and is a tracing of the original, showing a single contraction and relaxation at 20° C. Supramaximal stimulation was with a shock duration of 0.04 msec.

tion on the oscilloscope showed that this was by no means true. With ultra-violet light recordings the relaxation phase took four to eight times as long as the contraction phase and it was also characterized by the appearance of two distinct peaks or waves along its course. These waves are designated α and β for reference (Fig. 2). It may be seen from Fig. 2 that contraction begins sharply and increases uniformly until it reaches its maximum in approximately 0.2 sec. Contraction is followed immediately by the relaxation phase, which takes altogether between 0.8 and 1 sec, though sometimes it may take up to 1.5 sec. The α-wave appears about 0.3 sec after relaxation has begun and is followed a further 0.3 sec later by the β-wave. The descent to base-line after the β-wave is usually the longest part of the relaxation phase and takes about 0.5 sec on the average, though in Fig. 2 it is approximately 0.2 sec.

The characteristics of the α- and β-waves were not investigated in detail, but it was found that in the presence of high concentrations of potassium (5×10^{-3}), although contractions progressively diminished in size, the α-waves did not change appreciably and the β-waves became variable in shape, size and number.

If the α- and β-waves were absent they could be made to appear by increasing the frequency of stimulation or by the addition of acetylcholine to the bath. The more rapid the frequency of stimulation the more conspicuous were the α- and β-waves and the more quickly they appeared. If rapid stimulation (5 shocks/sec) was maintained for longer than 10 sec the whole of the relaxation phase became undulatory, though even then the α- and β-waves remained distinct.

When the α- and β-waves were evoked by high-frequency stimulation or by acetylcholine, resumption of stimulation at 0.45 shocks/sec and washing out the acetylcholine caused the disappearance of the waves within a few minutes.

To test whether the α- and β-waves were due to contraction of the smooth muscle in the skin, phenoxybenzamine (10^{-2}) was added to the bath before stimulation, but this drug did not abolish the appearance of these waves even after 30 min.

Electrical excitation: characterization of response

With supramaximal stimulation and a pulse duration of 0.04 msec or more, the preparation responded to single shocks with a single maximal contraction (Fig. 1). When the frequency of stimulation was increased to 0.5 shocks/sec relaxations became incomplete, but separate responses were still obtainable. At frequencies of 2 shocks/sec or higher, tetanic fusion occurred (Figs. 1 and 3).

The brief duration of the shock required to give maximal response made it likely that excitation was through the nerve fibres of the preparation. This view was supported by the results with tubocurarine and it seemed, therefore, that the disc of skin was a nerve-voluntary muscle preparation, and possibly also contained smooth muscle-nerve elements supplying the pilomotor system. To test this latter possibility the panniculus carnosus was removed from a disc of skin and the disc then set up in the bath and stimulated. Contractions were obtained which were, however, slower than those of the panniculus carnosus (Fig. 3). They were presumably due to the smooth muscle in the dermis, since they were completely abolished 20 min after adding phenoxybenzamine (10^{-2}).

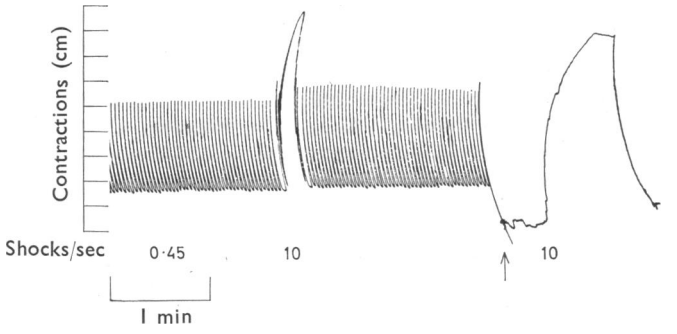

Fig. 3. Contractions of a rat skin preparation registered with a pen recorder. Ordinate scale as in Fig. 1. At the left the panniculus carnosus was intact. At the arrow the preparation was removed from the bath and the panniculus carnosus was dissected off. The skin was then remounted. Supramaximal stimulations with shocks of 0.04 msec duration were applied on the left of the arrow, and of 1 msec duration to the right.

Drugs

Tubocurarine. Usually stimulation of the panniculus carnosus with a shock duration of 0.04 msec is sufficient to produce a maximal response. When this response was completely blocked by tubocurarine (Fig. 4), increasing the shock duration to 0.07 msec gave small contractions and increasing the shock duration

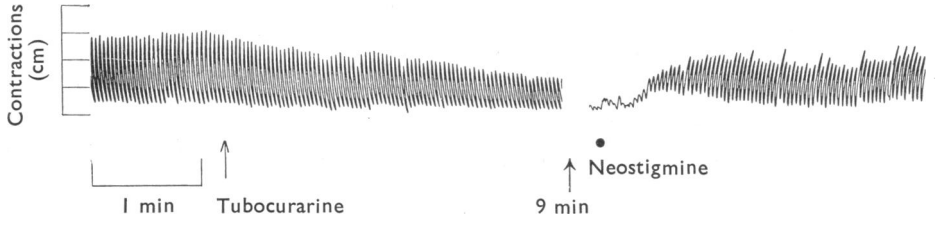

Fig. 4. Contractions of a rat panniculus carnosus preparation registered with a pen recorder. Ordinate scale as in Fig. 1. Response at 20° C to supramaximal stimulation with shocks of 0.04 msec duration. At the arrow tubocurarine (2×10^{-4}) and at ● neostigmine (10^{-5}) were applied. There was an interval of 9 min between records. In this illustration the contractions immediately following addition of neostigmine were smaller than before tubocurarine, but later they became larger than the control contractions.

further (to 0.15 msec) gave full contractions again. Thus some muscle fibres are stimulated directly with a shock duration of 0.07 msec and all the fibres when the shock duration reaches 0.15 msec.

The block produced by tubocurarine was reversed by neostigmine (10^{-5}). This concentration of neostigmine alone caused a 60% reduction in the size of the response, which was rapidly reversed on washing out the bath. During this inhibition the relaxation phase of the contractions became very undulatory as seen on the oscilloscope.

Decamethonium. Decamethonium (10^{-6}) did not inhibit the response to electrical excitation, but a concentration of 10^{-5} gave a 75% depression of the contractions after 5 min. This depression was accelerated in the presence of neostigmine (10^{-6}).

Acetylcholine. Acetylcholine (10^{-5}) in the presence of neostigmine (10^{-8}) caused an 85% reduction in the size of contractions, rapidly reversible on washing out the bath. Acetylcholine alone (10^{-10} to 10^{-5}) had no effect on the preparation or on the electrically stimulated contractions.

Potassium. Concentrations of potassium less than 10^{-3} were without effect on the contractions, but concentrations above this produced rapid block. This block was not overcome by an increase in the duration of the stimulating shock, though washing out the drug rapidly restored the excitability of the muscle (Fig. 5).

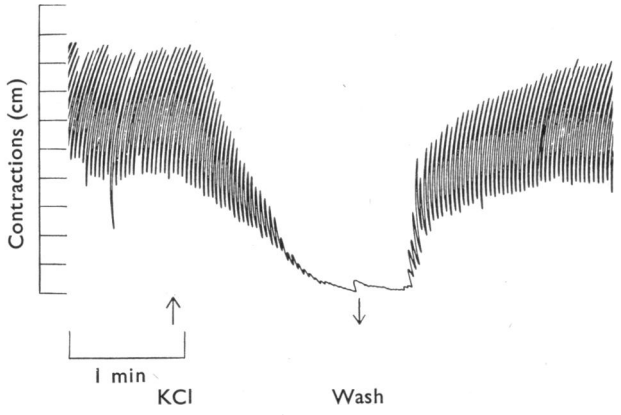

Fig. 5. Contractions of a rat panniculus carnosus preparation registered with a pen recorder. Ordinate scale as in Fig. 1. Response at 20° C to supramaximal stimulation with shocks of 0.04 msec duration. At the first arrow potassium chloride (4×10^{-3}) was added and at the second arrow it was washed out.

Adrenaline. Adrenaline (10^{-4}) did not contract the preparation and did not affect the usual contractions due to electrical stimulation. When the preparation was fatigued, however, an immediate increase in the height of the contractions produced by " direct " electrical stimulation (with a shock duration of 0.15 msec) occurred on addition of adrenaline. On washing out the adrenaline the contractions reverted to their previous size. Adrenaline had no effect on the contractions produced by " indirect " electrical stimulation (with a shock duration of less than 0.07 msec).

Histology

Transverse sections showed that there was some scattered smooth muscle in the dermis, probably associated with hair follicles, whilst the panniculus carnosus lay immediately below the dermis. The panniculus carnosus consisted of striated muscle and was about 0.25 mm thick. Longitudinal sections cut in the plane of the skin showed that the fibres were all orientated in the same direction.

DISCUSSION

A new method of transmural stimulation permitted an examination of the behaviour of the panniculus carnosus of rat skin. It is evident that this preparation could be employed as a simple alternative to the rat phrenic nerve-diaphragm preparation (Bülbring, 1946). Since from six to eight preparations can be obtained from one rat it is economical in use of animals, and since it can be easily set up in less than 2 min it is also economical in time.

The method of transmural stimulation used here would also be suitable for the investigation of smooth muscle in rat or any other skin. Indeed, it is obvious that the method could be applied to the investigation of a wide variety of problems in a number of tissues.

Preventing tissue asphyxia is, of course, a general problem for all *in vitro* preparations, but it is a particular problem for the usually relatively bulky mammalian skeletal muscle preparations. Dale & Gaddum (1930) attempted to overcome this difficulty by using kitten and rat diaphragms and Bülbring (1946) subsequently introduced the rat phrenic nerve-diaphragm as a pharmacological preparation.

Although the rat diaphragm can be used with the present method of stimulation it is not nearly so convenient as the panniculus carnosus. Moreover, the sensitivity of response which can be achieved with the panniculus carnosus is much greater than that when the rat phrenic nerve-diaphragm is used in the conventional way.

The accuracy of the records that can be obtained with the present method depends *inter alia* on the sensitivity of the recording apparatus available. Although recordings of the relaxation phase were different with pen and ultra-violet light recorders, the simpler contraction phase was qualitatively faithfully reproduced on the pen recorder, though almost certainly not quantitatively.

Although no convincing evidence can be offered here, it would seem from the behaviour of the α- and β-waves that they may reflect the " tone " of the preparation.

Responses to electrical stimulation showed some differences from those of other skeletal muscles in that tetanic fusions are not always followed by post-tetanic potentiation and by the appearance of the α- and β-waves in the relaxation phase.

Responses to drugs were, however, much as might be expected ; thus tubocurarine blocked indirect stimulation as it does in rat diaphragm (Holmes, Jenden & Taylor, 1951), though fairly large doses of neostigmine were required to reverse this effect. Acetylcholine had no effect when added to the bath. It might not be impossible, however, to give it into any one of the many prominent vessels which supply the panniculus carnosus and the results might well then be different. Adrenaline potentiated the contractions of the fatigued preparation, but not otherwise. This result resembles the findings of Bülbring (1946) in the rat isolated phrenic nerve-diaphragm preparation.

The blocking action of decamethonium developed rapidly and no contractures were seen in contrast to certain avian muscles such as the chick biventer cervicis (Ginsborg & Warriner, 1960) and the chick semispinalis (Child & Zaimis, 1960)

which respond to decamethonium with a true contracture. On the other hand, the panniculus carnosus of the rat appears to be about ten times more sensitive than the rat diaphragm (Thesleff, 1955) though it is very much less sensitive than the isolated cat tenuissimus (Maclagan, 1962). This result is perhaps not surprising in view of the great species differences in sensitivity to decamethonium which Paton & Zaimis (1949) found. Of the animals which they examined the rat was the least sensitive to decamethonium, while the cat was the most sensitive.

For excellent technical and electronic work I wish to thank Mr D. I. Duke and Mr J. A. Stedman, and for the illustrations I am indebted to Mr G. D. Leach. I am very much indebted to Dr J. R. Vane for his criticisms of this paper.

REFERENCES

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Brit. J. Pharmacol.*, **1**, 38–61.

CHILD, K. J. & ZAIMIS, E. (1960). A new biological method for the assay of depolarizing substances using the isolated semispinalis muscle of the chick. *Brit. J. Pharmacol.*, **15**, 412–416.

CLARK, W. E. LE G. (1958). *The Tissues of the Body*, 4th ed. Oxford: Clarendon Press.

DALE, H. H. & GADDUM, J. H. (1930). Reactions of denervated voluntary muscle, and their bearing on the mode of action of parasympathetic and related nerves. *J. Physiol. (Lond.)*, **70**, 109–144.

GINSBORG, B. L. & WARRINER, J. (1960). The isolated chick biventer cervicis nerve-muscle preparation. *Brit. J. Pharmacol.*, **15**, 410–411.

HELLMANN, K. (1963a). The isolated pilomotor muscles as an *in vitro* preparation. *J. Physiol. (Lond.)*, in the press.

HELLMANN, K. (1963b). Transmural stimulation of non-tubular tissues. *J. Physiol. (Lond.)*, in the press.

HELLMANN, K., STEDMAN, J. A. & DUKE, D. I. (1963). Assessment of functional competence in isolated muscular tissues. *Nature (Lond.)*, **199**, 494–495.

HOLMES, T. E. B., JENDEN, D. J. & TAYLOR, D. B. (1951). The analysis of the mode of action of curare on neuromuscular transmission: the effect of temperature changes. *J. Pharmacol. exp. Ther.*, **103**, 382–402.

MACLAGAN, J. (1962). A comparison of the responses of the tenuissimus muscle to neuromuscular blocking drugs *in vivo* and *in vitro*. *Brit. J. Pharmacol.*, **18**, 204–216.

PATON, W. D. M. & ZAIMIS, E. (1949). The pharmacological actions of polymethylene bistrimethylammonium salts. *Brit. J. Pharmacol.*, **4**, 381–400.

THESLEFF, S. (1955). The effects of acetylcholine, decamethonium and succinylcholine on neuromuscular transmission in the rat. *Acta physiol. scand.*, **34**, 389–392.