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ON THE PHARMACOLOGICAL AND ANAPHYLACTIC RESPONSIVENESS OF DENERVATED SKELETAL MUSCLE OF THE GUINEA-PIG

BY

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We have recently reported (Alonso-deFlorida, del Castillo, Gonzalez & Sanchez, 1965) that chronically denervated diaphragmatic muscle of the guinea-pig not only becomes supersensitive to acetylcholine but also exhibits a considerable and unexpected sensitivity to histamine and bradykinin. Moreover, strips of denervated diaphragm taken from sensitized guinea-pigs contract in the presence of small concentrations of the homologous antigen, a finding which largely confirms the experiments of Ado & Ginetsinskii (1944) and Ado, Ginetsinskii & Shamarina (1946) on the allergic reaction of denervated muscles of the dog *in situ*.

These results are interesting for two main reasons. First, they show that denervated guinea-pig diaphragm develops chemical receptors to compounds which are inactive before the degeneration of the phrenic nerve; this finding offers a fresh opportunity to probe into the mechanisms by which the motor nerve controls the chemical sensitivity of the muscle membrane. Second, the fact that skeletal muscle can show anaphylactic responses is important technically, since striated muscle fibres are larger than smooth muscle cells and more suitable for the study of the changes in membrane permeability and electrical properties elicited by the antigen on sensitized tissues.

This paper describes the responses of denervated muscle strips to histamine and antigenic proteins. The effect of histamine is compared with that of acetylcholine; our investigation of the effects of the homologous antigen follows the lines of the classical work of Schultz (1910) and Dale (1913) on the *in vitro* anaphylactic responses of visceral smooth muscle.

METHODS

The left denervated hemidiaphragms of over 100 guinea-pigs have been used in these experiments. Most of the animals were young, weighing less than 300 g, as the *in vitro* survival and functional condition of the strips of diaphragmatic muscle appear to be inversely related to their thickness.

The left phrenic nerve was cut in the cervical region during pentobarbitone sodium anaesthesia, and 1 week after denervation active immunization was begun by injecting antigenic proteins according to three different schedules. (1) Most of the animals were immunized to ovalbumin, human serum-albumin or

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diazotized human serum-albumin with a single subcutaneous injection of 10 mg of protein in complete Freund adjuvant. (2) In an attempt to increase the anaphylactic responses, a number of animals were sensitized to ovalbumin with a subcutaneous injection of 20 mg of the protein in complete Freund adjuvant followed, at intervals of 1 week, by two intracutaneous injections of 0.1 ml. of an alum-precipitated protein suspension (10 mg/ml.). However, the results did not differ significantly from those obtained with a single injection. (3) Finally, it was observed that anaphylactic responses to ferritin could only be obtained after a subcutaneous injection of 20 mg of this protein in complete Freund adjuvant followed by seven injections once per day of 0.1 ml. of an alum-precipitated suspension (10 mg/ml.).

At 2 to 6 weeks after denervation, and at least 1 week after the last injection of antigen, the animals were killed and the left hemidiaphragm was excised and divided by cuts parallel to the direction of the muscle fibres. The resulting strips, about 5 mm in width and from 9 to 13 mm in length, were kept for at least 1 hr before the experiments in warm, oxygenated Krebs solution. The effects of drugs and antigens were recorded by attaching the muscle strips to an isotonic lever writing with a magnification of $\times 15$ on a smoked drum. A tension of 1 g was applied to all preparations.

Muscle strips taken from the right innervated hemidiaphragms of sensitized and nonsensitized animals and from the left denervated hemidiaphragms of nonsensitized guinea-pigs were used as controls.

The Krebs solution used had the following ionic composition (mM): Na, 142.90; K, 5.88; Ca, 1.26; Mg, 1.18; Cl, 125.22; HCO_3, 24.90; SO_4, 1.18; and H_2PO_4, 1.18. A mixture of 98% oxygen and 2% carbon dioxide was continuously bubbled through the solution, which was maintained at a temperature of 39° C.

Dose/response curves to histamine and acetylcholine were drawn from values obtained by the cumulative technique described by Ariëns & de Groot (1954) (see also Ariëns, Simonis & van Rossum, 1964). Successive amounts of the drug were added to the bath, doubling the resulting concentration each time. The contractions elicited by each dose appear superimposed on the peak of the contraction caused by the previous one. The concentration was increased until the shortening of the muscle reached a plateau. This level was used to express the amplitudes of the previous contractions.

The possibility of acetylcholine being released in the denervated diaphragm of immunized guinea-pigs under the influence of the antigen was explored as follows: the diaphragm of a denervated, sensitized animal was divided into two halves, an innervated (I) and a denervated (D) one. Each half was minced and incubated in oxygenated Krebs solution containing physostigmine (1 μg/ml.) at 39° C for 1 hr. Each sample, I and D, was then divided into two equal parts (I1, I2, D1 and D2). Homologous antigen (ovalbumin, 100 μg/ml.) was added to I1 and D1, while I2 and D2 served as controls. The supernatant fluid from each sample was tested for acetylcholine on the frog rectus abdominis muscle preparation in the presence of physostigmine. Contractions were elicited in this preparation by control acetylcholine solutions at a minimum concentration of 0.1 μg/ml.

RESULTS

Spontaneous mechanical activity

When observed under a binocular microscope, strips of denervated guinea-pig diaphragm exhibited continuous twitching of individual fibres and small groups of fibres. This fibrillation may last for several hours provided adequate oxygenation of the Krebs solution is maintained.

Due to its random character, fibrillatory activity does not usually result in rapid contractions of the whole muscle. Yet, most preparations have a certain degree of tonus, as can be shown by cooling the surrounding Krebs solution which causes the muscle to relax. Though the influence of the temperature on tonus has not been investigated systematically, maximal relaxation occurred below about 21° C. Another way of showing the occurrence of such steady tonic contraction is to apply acetylcholine (in concentrations of 3 μg/ml. and higher) and allow it to remain in the bath. After the peak of the contraction a rapid relaxation takes place which brings the trace to a level well below the initial baseline (Fig. 5,*f*).

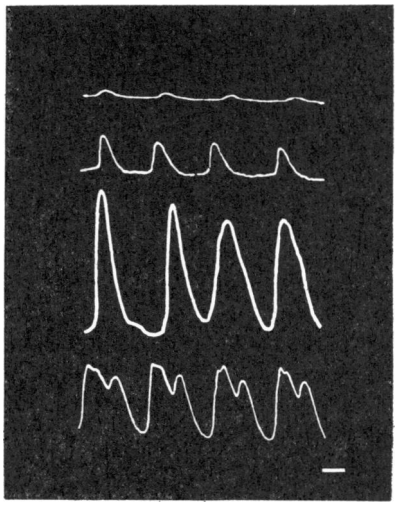

Fig. 1. Spontaneous mechanical activity in strips of denervated guinea-pig diaphragm, at 39° C. Time calibration, 1 min. The strips came from four different animals.

In approximately 10% of the preparations, waves of contraction were seen to alternate rhythmically with periods of relaxation at a frequency of between 0.5 and 1 cycles/min. This type of activity was observed only in muscle strips kept for at least an hour in vigorously oxygenated Krebs solution at 39° C (Fig. 1). These contractions were usually smooth showing a single peak, although more complex mechanograms formed, presumably, from the sum of two or more separate waves, were also observed (lower trace of Fig. 1). The amplitude of the spontaneous contractions varied in different preparations even among those taken from the same hemidiaphragm; in several instances, they were comparable in amplitude with those elicited by 1 μg/ml. of acetylcholine.

Effects of histamine and acetylcholine

Denervated muscle strips contract upon the addition of histamine and acetylcholine to the bath. The effective concentrations of these two compounds are similar, as shown by the dose/response curves in Fig. 2.

As Ariëns *et al.* (1964) claimed, and it was confirmed in our experiments, the points plotted from cumulative dosage experiments show a smaller scatter than the measurements obtained by conventional methods. Besides the reasons given by these authors, the smaller deviations may also be due to the fact that measurements were made within a relatively short period, during which the condition of the preparation does not change significantly. One should emphasize, however, that the amplitude of the contractions elicited by the cumulative concentrations of either histamine or acetylcholine were smaller at every dose level, except the first one, than those seen when the preparations were exposed to separate doses of these compounds in different tests. For example, trace (*a*) in Fig. 3 shows that the response of a denervated muscle strip to 2 μg/ml. of acetylcholine is much larger than the contraction produced when the same concentration was attained cumulatively (*b*). Nevertheless, we were unable to show any significant differences in the slope of the approximately linear region of the dose/response curves obtained with both methods from the same preparation.

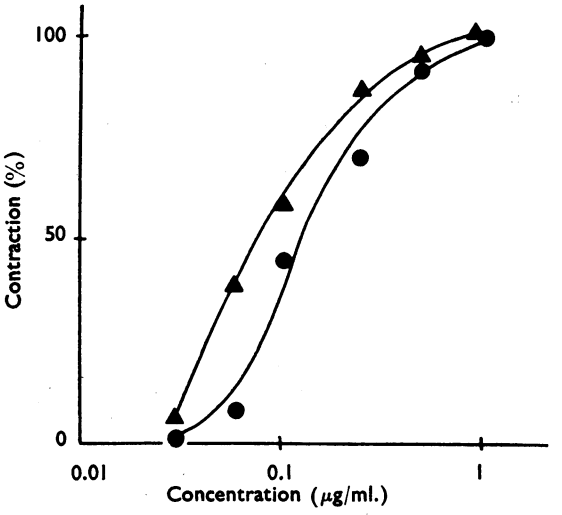

Fig. 2. Dose/response curves to histamine (▲) and acetylcholine (●). Abscissa: concentration of the drugs in μg/ml. Ordinate: percentage of maximal contraction. The molecular weights of the salts (histamine dihydrochloride and acetylcholine chloride) differ by less than 2%.

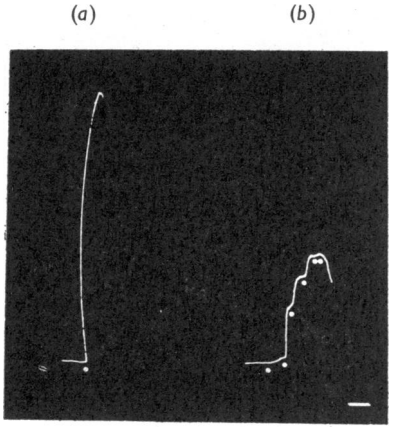

Fig. 3. (*a*) Contraction of a strip of denervated guinea-pig diaphragm on addition of acetylcholine to the bath; final concentration, 2 μg/ml. (*b*) Increasing shortening elicited in the same preparation by the following cumulative concentrations of acetylcholine: 0.06, 0.12, 0.25, 0.5, 1 and 2 μg/ml. Time calibration, 1 min. Acetylcholine added at white dots.

Although the amplitudes of the contractions elicited by equimolar concentrations of histamine and acetylcholine were approximately the same, two interesting differences were observed between the effects of these two compounds. In the first place, the contraction caused by the administration of acetylcholine develops almost immediately whereas the shortening produced by histamine always begins after a latent period of about 10 sec. Secondly, the peak of the contraction induced by acetylcholine is followed by a relatively rapid relaxation but the shortening produced by histamine may last several minutes. Often, after a partial relaxation, the muscle contracts again (Fig. 5,*b*).

Anaphylactic contraction of denervated muscle strips

The administration of ovalbumin to denervated preparations taken from animals sensitized to this protein produces a contraction. If the preparation is now thoroughly washed with fresh Krebs solution and a second dose of protein is given after 5 or 10 min a smaller contraction is usually observed (Fig. 4, uppermost traces). In some preparations, however, the second dose of antigen fails to elicit any mechanical response at all (Fig. 4, lower traces). In general, the higher the concentration of the first dose of antigen, the smaller is the response to the second dose (Fig. 4). The same is true for the contractions elicited by the second and third doses of protein.

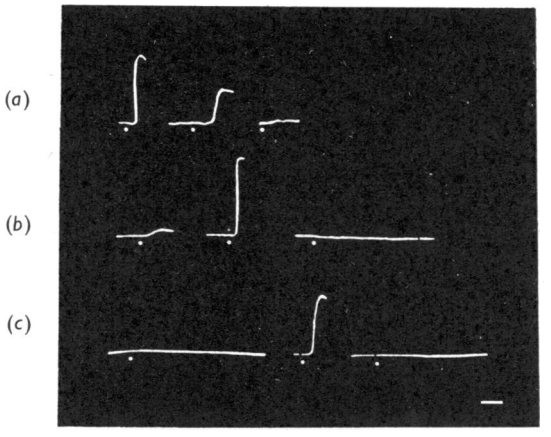

Fig. 4. Responses elicited in three different strips of denervated diaphragm taken from guinea-pigs actively sensitized to ovalbumin. (*a*, uppermost traces) three doses of ovalbumin were added at intervals of 6 min. Final bath concentrations, from left to right respectively, were 10, 100 and 100 μg/ml. The preparation was thoroughly washed between each application. Notice increased latency and decreased amplitude and rate of shortening in the second response. (*b*, middle traces) From left to right: responses to the addition of human serum-albumin (100 μg/ml.) and two doses of ovalbumin (100 μg/ml.). Notice small contraction produced by human serum-albumin and the rapid desensitization following the first dose of ovalbumin. (*c*, lowest traces) From left to right: a dose of ferritin (100 μg/ml.) followed by two doses of ovalbumin (100 μg/ml.). Time calibration, 1 min.

A latent period of about 30 sec is always observed between the first administration of antigen to the bath and the onset of the anaphylactic contraction. This latent period increases, and the rate of shortening is seen to decrease, in the responses elicited by subsequent doses of antigen, as illustrated in the uppermost traces of Fig. 4.

After the mechanical responses are abolished by the initial administration of several successive doses of antigen, the anaphylactic sensitivity is partially restored in some instances after thoroughly washing the preparation with fresh saline and allowing it to rest at 39° C for at least 1 hr (Fig. 5). A second administration of antigen now produces very small responses and a fast and irreversible desensitization.

Denervated muscle strips taken from animals sensitized to either ferritin or diazotized human serum-albumin also respond with a contraction to the addition of homologous antigen (Fig. 6). However, the contractions elicited by ferritin are always smaller and

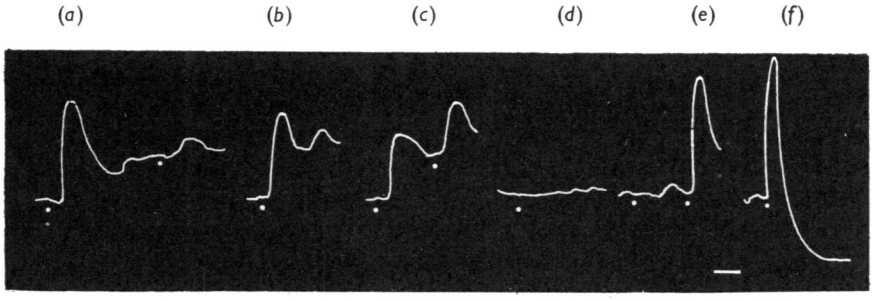

Fig. 5. Responses of a strip of denervated diaphragm taken from a guinea-pig sensitized to ovalbumin. (*a*) First dot indicates the addition of ovalbumin, 100 μg/ml. followed, without washing the preparation, by histamine (3 μg/ml.), at second dot. (*b*) Histamine (3 μg/ml.) applied 60 min after (*a*). (*c*) Ovalbumin (100 μg/ml.) applied 60 min after (*b*), at first dot. Histamine (3 μg/ml.) was added without washing the preparation, at second dot. (*d*) Ovalbumin (100 μg/ml.) applied 15 min after (*c*). (*e*) Ovalbumin (100 μg/ml.) applied 60 min after (*d*), at first dot. Second dot indicates the addition of histamine (3 μg/ml.). (*f*) Acetylcholine (3 μg/ml.); notice relaxation to a level well below the initial baseline. These records show that the amplitude of the response to histamine added in the presence of the homologous antigen seems to be an inverse function of the degree of shortening induced by the protein. This relation has been observed in many experiments.

develop after longer latent periods than those caused by ovalbumin or diazotized serum-albumin. It should also be emphasized that, to obtain any anaphylactic responses at all to ferritin, the prolonged technique of sensitization described in Methods is necessary.

Anaphylactic contractions are also produced by the administration of proteins structurally related to the homologous antigen. Thus, human serum-albumin causes the contraction of denervated muscle strips taken from animals sensitized to ovalbumin (Fig. 4, middle traces). The size of these contractions varies within wide limits and they are usually slower than those caused by the homologous antigen. However, ferritin and diazotized human serum-albumin failed to produce any effects on preparations taken from

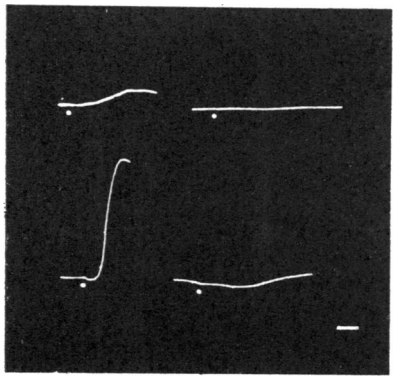

Fig. 6. Responses of a strip of denervated diaphragm taken from a guinea-pig sensitized to ferritin (upper records) and from one sensitized to diazotized human serum-albumin (lower records) to the administration of the homologous antigen. All the concentrations were 100 μg/ml. Notice small size and rapid desensitization of the responses elicited by ferritin. Time calibration, 1 min.

animals sensitized to egg albumin (Fig. 4, lowest traces). Furthermore, preparations taken from animals sensitized to ferritin failed to respond to any of the other proteins used in this work.

The very long latencies of the responses and the progressive degree of desensitization produced by successive doses of the antigen precluded the use of the cumulative dosage technique to determine the dose/response curves for the anaphylactic contraction and made it necessary to use separate preparations to determine each experimental point. To plot these curves the denervated hemidiaphragms were divided into six muscle strips and a single different dose of antigen was applied separately to each preparation. Concentrations of ovalbumin of 1, 3, 10, 30, 100 and 300 μg/ml. were used.

Fig. 7 shows a dose/response curve plotted by this method. The amplitude of the responses is expressed in terms of the contraction produced by a constant concentration of acetylcholine. This and other curves showed that the amplitude of the contraction is maximal at protein concentrations of either 30 or 100 μg/ml.

Control experiments on denervated hemidiaphragms of nonsensitized animals and the intact, innervated hemidiaphragms of both sensitized and nonsensitized animals gave negative results. All these muscles proved to be completely insensitive to all the proteins used in this investigation.

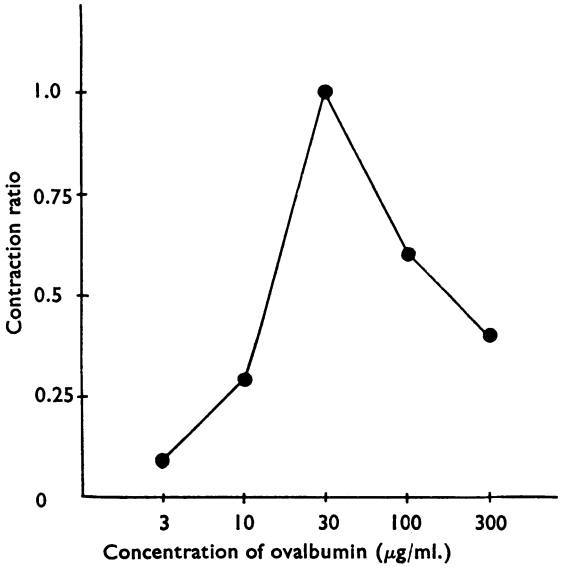

Fig. 7. Relation between the concentration of homologous antigen (ovalbumin) and the amplitude of the contractions elicited in strips of denervated, sensitized guinea-pig diaphragm. Abscissa: concentration of ovalbumin (μg/ml.). Ordinate: ratio between the contraction elicited by ovalbumin and that by acetylcholine (3 μg/ml.).

Effect of tubocurarine, tripelennamine and physostigmine on the anaphylactic contraction of denervated muscle

In view of the role attributed to histamine in the anaphylactic reactions of the guinea-pig muscle and the suggestion advanced by Ado *et al.* (1946) that acetylcholine may be the

mediator of the anaphylactic reactions in mammalian muscle, experiments were performed to test the influence of the above compounds on the contractions which follow the administration of the homologous antigen.

As was to be expected, tubocurarine (5 μg/ml.) completely blocks the contraction elicited by acetylcholine. However, even concentrations of tubocurarine as high as 0.1 mg/ml. do not block the action of the homologous antigen on immunized muscle strips nor the contractile responses elicited by histamine.

Tripelennamine, at a concentration of 1 μg/ml., blocks the responses both to histamine (1 μg/ml.) and to ovalbumin (30 and 100 μg/ml.) but it does not affect the contractions elicited by acetylcholine.

Failure to show acetylcholine release in denervated muscle upon addition of antigen

Experiments performed, as described in Methods, to see whether the addition of the homologous antigen to minced diaphragmatic tissue, both normal and denervated, causes the liberation of acetylcholine, gave completely negative results.

DISCUSSION

Responses to antigenic proteins

The contractions produced by the homologous antigen on denervated muscle from sensitized guinea-pigs appear to have an immunological origin and to show all the characteristic features of the Schultz-Dale reaction of visceral smooth muscle: they were observed only in preparations taken from sensitized animals; they were elicited only by the homologous antigen or structurally related proteins; the latent periods preceding the responses to antigen were longer than those which follow the application of pharmacologically active compounds; desensitization produced by repeated antigen administration was only partially reversible; and the dose/response curves were bell-shaped.

Intracellular recording from muscle fibres of denervated diaphragmatic muscle during the anaphylactic responses has shown (Alonso-deFlorida & del Castillo, unpublished) that shortening is caused by long bursts of action potentials which begin suddenly about 30 sec after the addition of the antigenic protein to the bath and may last for periods of up to 1 to 3 min. These observations justify the use of the term contraction, instead of contracture, with reference to such responses. Preliminary experiments with histamine have given similar results.

Role of acetylcholine in the anaphylactic reaction of denervated muscle

Ado *et al.* (1946) suggested that the contraction of denervated muscles caused by intra-arterial injection of antigen might be due to acetylcholine liberation. This hypothesis, however, received no support from our experiments.

We have seen, for instance, that: shortening induced by the antigen can be sustained at a reduced level during relatively long periods (Fig.5) whereas the same muscle strips rapidly relax in the continued presence of acetylcholine; the responses to histamine and homologous antigen were blocked by tripelennamine at concentrations which did not interfere with the effects of acetylcholine; although tubocurarine completely abolished the responses

to acetylcholine it did not affect the contractions elicited by either histamine or antigen; we failed to demonstrate liberation of acetylcholine from diaphragmatic tissue under the influence of the antigen.

Development of new histamine receptors

The mechanical responses elicited by adrenaline on chronically denervated skeletal muscle (Euler & Gaddum, 1931; Luco & Sanchez, 1956; Boohla & Schachter, 1961) have been interpreted (Sharpless, 1964) as the result of a qualitative change in the chemical sensitivity of the muscle membrane. However, the observations of Bülbring & Burn (1940), Brown, Bülbring & Burns (1948) and Goffart & Ritchie (1952) show that normal innervated muscle does have receptors to adrenaline, since this compound increases the duration of the muscle action potential, decreases the rate of conduction and potentiates maximal twitch tension.

Our experiments on the effect of histamine on denervated guinea-pig diaphragm may be regarded, therefore, as a more convincing proof that, besides the usual supersensitivity to the physiological synaptic transmitter and other compounds active before the degeneration of the motor nerve, denervated muscle may also develop qualitatively new chemical receptors.

This phenomenon appears to be related to the species used. Thus, the denervated diaphragm of the rat fails to develop receptors to either histamine or bradykinin (Boohla & Schachter, 1961) while it is known from the work of Dale & Gasser (1926) and Dale & Gaddum (1930) that denervated gastrocnemius and diaphragm muscles of the cat do not become sensitive to histamine. It should be emphasized, however, that, although histamine has no obvious effects on normal striated muscle, this compound is known to exert a stimulating action on frog's muscle immersed in calcium-poor solution (Bülbring, 1955).

The development of functional receptors to histamine in the denervated guinea-pig diaphragm could be accounted for by two different hypotheses: the receptors may be present in the muscle surface membrane before denervation, but inhibited by a factor released by the nerve terminals (Axelsson & Thesleff, 1959); or new receptors may arise due to the attachment of circulating molecules to the membrane of the denervated muscle fibres, a process which might be normally hindered by a nerve factor. These possibilities are now being explored in this laboratory. Another interesting question is whether the emergence of new histamine receptors is coupled with the spread of acetylcholine receptors from the end-plate region known to occur after denervation (Miledi, 1962).

Spontaneous mechanical activity

An incidental but interesting question posed by our experiments concerns the mechanisms responsible for the spontaneous rhythmic contractions shown by strips of denervated guinea-pig diaphragm. It is difficult to believe that such large and regular contractions could be possible in the absence of mechanisms synchronizing the activity of individual muscle fibres. Electrical and mechanical interactions are possible in principle, and observations made in this laboratory suggest that both types are likely to exist. Thus, current injected into a fibre gives rise to small electrotonic potentials in adjacent fibres. Furthermore, the membrane potential of denervated muscle fibres in the same preparations has proved to be extremely sensitive to mechanical disturbances (Alonso-deFlorida & del Castillo, unpublished).

SUMMARY

1. The effects of histamine and antigenic proteins on strips of denervated guinea-pig diaphragm kept in oxygenated Krebs solution at 39° C were studied.

2. These preparations exhibit a conspicuous mechanical activity. Most of them have an appreciable degree of tonus and some show large rhythmic contractions.

3. Addition of histamine (1 to 3 μg/ml.) to the bath elicits a contraction similar in amplitude to those produced by equimolar concentrations of acetylcholine.

4. The effect of acetylcholine is immediate and always followed by relaxation; conversely, the shortening elicited by histamine begins after a latent period of about 10 sec, and can last several minutes.

5. Preparations taken from actively sensitized guinea-pigs contract upon the addition of homologous antigen or structurally related proteins. These responses are followed by a rapid and irreversible desensitization.

6. Dose/response curves plotted from preparations sensitized to ovalbumin are bell-shaped. Maximal contraction occurs at protein concentrations of either 30 or 100 μg/ml.

7. The responses elicited by the antigen are blocked by tripelennamine (1 μg/ml.) but are not affected by tubocurarine (up to 5 μg/ml.).

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