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A CHOLINOCEPTOR ANTISERUM: ITS PHARMACOLOGICAL PROPERTIES

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1 Sera of rabbits immunized against a nicotinic receptor-rich fraction, obtained from the electric organ of *Torpedo marmorata*, were tested for their pharmacological activity on different *in vitro* preparations.

2 Sera containing antibodies against the nicotinic receptor blocked neuromuscular transmission in the phrenic-nerve hemidiaphragm preparation without affecting the muscle responses evoked by direct electrical stimulation. Complement inactivated sera were still active. Immune sera, incubated for 15 min with a receptor-rich fraction, lost their activity.

3 The immune sera antagonized the responses elicited by acetylcholine on the frog rectus abdominis.

4 The immune sera tested *in vitro* decreased the compound action potential evoked in the superior cervical ganglion of the rat by electrical stimulation of the preganglionic nerve.

5 The sera did not show any activity on muscarinic receptors of the guinea-pig ileum preparation.

6 It is concluded that in sera obtained from immunized rabbits a substance is present with curare-like action, and that this activity is probably due to the presence of antibodies against the nicotinic receptor.

Introduction

It has been demonstrated by Patrick & Lindstrom (1973) that repeated injections of purified *Torpedo* acetylcholine receptors into rabbits induce a flaccid paralysis which has some similarities to human myasthenia gravis. It was assumed that the development of this flaccid paralysis might be due to the production of antibodies against the cholinoceptor protein, and that such antibodies could partially inactivate the rabbit's own cholinoceptors. This experimental model may represent a valid approach in clarifying the pathogenesis of human myasthenia gravis and may support the hypothesis that in this illness the impairment of neuromuscular transmission, and probably of the chemoreceptor of the muscle end-plate (Johns, Grob & Harvey, 1965; Grob, Namba & Feldman, 1966) may be due to an immunological disturbance (Nastuck & Plescia, 1966; Feltkamp, Van Den Berg-Loonen, Nijenhuis, Engelfriet, Van Rossum, Van Loghem & Oosterhuis, 1974). This is consistent with the presence of neuromuscular blocking agents in the sera of myasthenic patients (Berg, 1953; Nastuck, Strauss & Osserman, 1959; Walker, 1973; Laurent, 1973), with the recent findings that have demonstrated a decreased number of active receptor-sites in myasthenic muscles (Fambrough, Drachman & Satyamurti, 1973) and with the presence in myasthenic subjects of a serum globulin which inhibits the binding of α-bungarotoxin to acetylcholine receptors (Alman, Andrew & Appel, 1974). Recently we have produced in rabbits a muscle relaxation that was longer lasting than that previously obtained, and with more similarities to human myasthenia gravis, by immunizing the animals with a cholinoceptor-rich fraction isolated from *Torpedo* electric organs (Berti, Clementi, Conti-Tronconi & Omini, 1974). The results described in this paper demonstrate that a factor which is able to block the nicotinic receptor *in vitro* in a variety of tissues, is present in the serum of such immunized animals.

Methods

Specimens of *Torpedo marmorata* were kindly supplied by Dr Martin, Zoological Station, Naples, and kept in artificial oxygenated sea water at 18–20°C. The electric organs were fractionated according to Cohen, Weber, Huchet & Changeux (1972) as reported in detail elsewhere (Clementi, Conti-Tronconi, Berti & Folco, 1976.

One ml of cholinoceptor-rich fraction containing 1 mg protein was emulsified with 1 ml of complete Freund adjuvant and injected in 10 spots on the back of male albino rabbits (3.0–3.5 kg body weight). The injection was repeated 15 days later and a third dose was given to those animals which did not show muscle

weakness. Sterile 0.9% w/v NaCl solution (saline) (40 ml) containing 5% glucose was given subcutaneously, daily, to the treated animals.

Blood was obtained from rabbits by cardiac puncture without anticoagulant 7–10 days from the development of muscle weakness. The blood was allowed to clot at room temperature for 30 min and the serum was separated from blood cells by centrifugation and then stored in a deep freeze. Only sera that gave clear precipitation lines between the undiluted serum and the solubilized receptor protein on immunoassay (Clementi *et al.*, 1975) were used for pharmacological analysis.

Normal and immune sera, diluted in appropriate salt solution, were assayed for their pharmacological activities on a series of *in vitro* preparations. Complement was inactivated by heating the sera at 56°C for 30 minutes.

Rat isolated phrenic nerve-diaphragm

The left hemidiaphragm of male albino rats (Wistar, 180–200 g body weight) was suspended in a 50 ml bath containing Krebs solution according to Bülbring (1946) and bubbled with 95% O_2 and 5% CO_2. The phrenic nerve was stimulated by means of a Grass stimulator with rectangular pulses of 1 ms duration at a frequency of 0.1 Hz and three times the strength necessary to produce a maximal twitch. Direct stimulation was applied between two silver pins embedded in the muscle near its origin in the ribs. Twitches were elicited by stimulation at a frequency of 0.1 Hz, 1 ms duration, and of a strength to produce contractions equal in amplitude to maximal twitches produced by indirect stimulation. The contractions of the muscle were recorded with an isometric transducer.

Frog rectus abdominis

Pithed frogs (*Rana pipiens*) weighing 20–40 g were used and the rectus abdominis muscle was isolated and suspended in a 10 ml bath as described by Van Maanen (1950). The contractions of the muscle were recorded with an isometric transducer.

Rat isolated superior cervical ganglion

Male Wistar rats (70–80 g body weight) were anaesthetized with ethyl-urethane and their right superior cervical ganglion was carefully dissected free. The ganglia, peeled from their connective capsule, were placed, according to Perri, Sacchi & Casella (1970) in a perspex chamber filled with a continuously flowing Krebs solution at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The ganglion cells were excited orthodromically by stimulation of the sympathetic trunk, with silver/silver chloride electrodes. Supramaximal square wave pulses of 0.3 ms duration from a Tektronix generator were applied, via an isolation unit, to the stimulating electrode at frequencies ranging from 1 to 10 Hz. The potentials derived from the internal carotid nerve were led directly to a Tektronix R 5103 N oscilloscope and recorded by a Grass Kymograph camera model C 4PR.

Guinea-pig ileum

Segments of terminal ileum from male guinea-pigs (350–500 g body weight) were placed in a 10 ml organ bath containing Krebs solution gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. Tonus and contractions of the organ were recorded by an isometric transducer.

Results

Effects of the immune serum on neuromuscular junction

The normal rabbit serum, with and without complement, at concentrations of up to 2% in the bath had no effect on the electrically evoked contractions of the rat hemidiaphragm.

Addition to the bath of the immune serum induced, after a delay of 15–20 min, a clear and progressive inhibition of the hemidiaphragm responses to the electrical stimulation of the phrenic nerve (Figure 1). However, the muscle responses elicited by direct electrical stimulation were not affected (Figure 1c). Immune sera obtained from different animals had different potencies in reducing the strength of the muscle contractions. The majority of the sera tested were active at concentrations of about 1%. Physostigmine induced a temporary partial reversal of the effect of the serum (Figure 1d) which was followed in some cases, by an enhancement of the block (Figure 1e). In the same preparations a further dose of physostigmine at higher concentration was also ineffective. This result could be explained by a presynaptic effect of physostigmine. Anticholinesterase drugs can induce a hyperpolarization of nerve endings (Bowman & Webb, 1972) which could impair the acetylcholine release. It is also possible that an excessive concentration of the preserved acetylcholine at the motor end plate may provoke a desensitization of the cholinoceptors (Katz & Thesleff, 1957). This phenomenon might be further enhanced by the action of antibodies on the receptors.

The effect of the sera was irreversible when its contact time with the hemidiaphragm was longer than 15 min (Figure 1d).

Inactivation of the complement of the immune sera

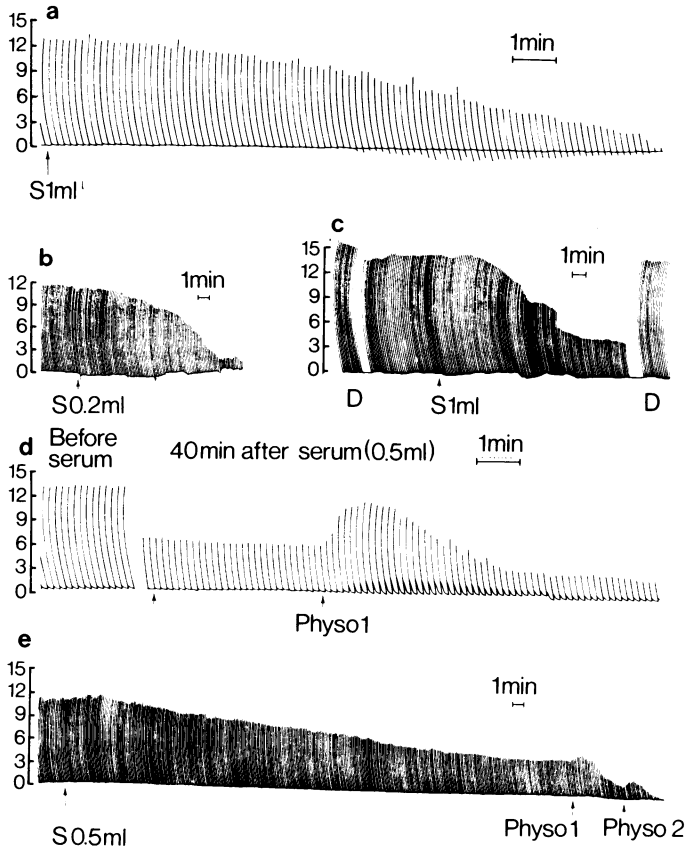

Figure 1 Effect and potency of different immune sera (S) on the rat hemidiaphragm preparation. The strength of the contractions is calibrated in grams. (a) Effect of immune serum No. 1 heated for 30 min at 56°C. (b) Effect of serum No. 2 showing a greater blocking effect. (c) Effect of immune serum No. 3. Twitches were elicited by direct (D) and indirect stimulation. The serum reduced the indirectly evoked responses without affecting significantly the responses induced by direct stimulation of the muscle. (d) Effect of physostigmine (Physo) on partially impaired contractions of rat hemidiaphragm by immune serum No. 4. Physostigmine (2.6 μM) was added to the organ bath 45 min after serum and 5 min after washing (arrow). (e) This recording demonstrates the enhancement of the blockade by physostigmine. Physo 1 = Physostigmine 2.6 μM, Physo 2 = physostigmine 13 μM.

by heating it at 56°C for 30 min did not influence their blocking activity (Figure 1a).

In order to understand whether the inhibitory activity of the immune serum was really due to its content of antibodies against the cholinoceptors, a mixture of 1 ml of active serum with 0.5 mg of receptor protein was prepared and tested 15 min later on the nerve-muscle preparation: no activity remained in such neutralized serum.

On frog rectus abdominis the immune serum displayed a curare-like activity, antagonizing, at concentrations ranging from 1% to 10%, the contraction produced by acetylcholine (Figure 2). Control sera did not show any effect on acetylcholine-induced contractions. Normal and immune sera had the same cholinesterase activity (Clementi, Conti-Tronconi, Berti & Omini, unpublished observations).

Effects of immune serum on ganglionic transmission

Since the sera were obtained by immunizing the rabbits against a nicotinic receptor, experiments were performed to investigate the specificity of the antibodies produced. It is known that nicotinic receptors are subdivided into at least two groups, since there are drugs that selectively interfere with transmission at ganglia, and other drugs which selectively inhibit neuromuscular transmission.

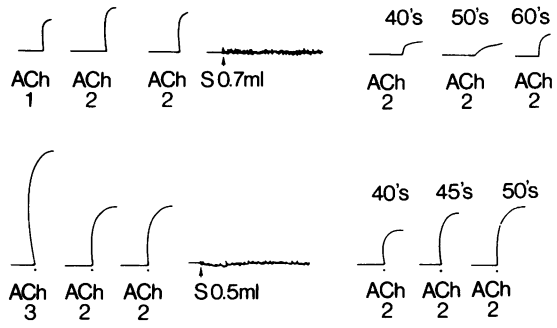

Figure 2 Serum No. 2 (S) upper panel and No. 1 (S) lower panel, show a clear antagonism, with different potencies, of the acetylcholine-induced contractions of the frog rectus abdominis preparation. The times quoted indicate the time after the addition of the serum to the preparations. Preliminary experiments showed that after 40 min of contact, the serum-induced inhibition was maximal. (The bath was washed after each response to acetylcholine). ACh1 = acetylcholine 3.4 μM, ACh2 = acetylcholine 6.8 μM, ACh3 = acetylcholine concentration 13.6 μM.

Figure 3 Recordings of compound action potential of rat superior cervical ganglion *in vitro*. The action potentials were recorded from the internal carotid nerve. (a) Control preparation. (b) Same preparation bathed 15 min with Ringer containing 5% of serum No. 2. (c) Same preparation 20 min after washing in normal Ringer. Calibration pulses are 1 mV and time marks indicate in (a) and (c) 10 ms and in (b) 20 ms.

The immune serum was tested on the isolated superior cervical ganglion of the rat. The immune sera at a concentration of 1% reduced the amplitude of the compound action potentials evoked in the superior cervical ganglion by electrical stimulation of the preganglionic trunk by 50% in about 15 min (Figure 3). Increasing the dose or the exposure time of the preparation to the serum did not lead to an increased inhibitory effect. The effect of the serum was completely removed by prolonged washing of the preparation, in contrast to the irreversible effect on the neuromuscular junction.

Effects on muscarinic receptors

When the immune sera were tested on guinea-pig ileum preparations, no inhibitory activity against spontaneous motility or acetylcholine-induced contraction was detected. However several sera, both normal and immune, induced a sustained contraction of the isolated ileum which was not antagonized by atropine. Inactivation of the complement did not alter the serum activity.

Discussion

The pharmacological analysis of sera obtained from rabbits that developed a muscular weakness after various injections of *Torpedo* acetylcholine receptor-rich fraction indicates the presence in the sera of a factor which interferes with neuromuscular transmission. In this respect the activity of the immune serum shows some similarities with curare-like drugs. This assumption is supported by the fact that on rat hemidiaphragm preparations the immune sera impaired neuromuscular transmission without affecting the muscular contractions evoked by direct stimulation. Furthermore the blockade induced by the sera could be temporarily antagonized by anti-cholinesterase drugs.

In addition, the antagonism exerted by immune sera against the depolarizing effect of acetylcholine on frog rectus abdominis further emphasizes the antinicotinic receptor activity of the immune serum. The effect of the immune sera seems to be specific against nicotinic receptors, since the muscarinic receptors on the ileal smooth muscle are not affected.

The reduction of the compound action potentials evoked in the superior cervical ganglion of the rat by the immune serum is only partial and is reversible, providing further evidence of steric differences between nicotinic receptors in ganglia and at the neuromuscular junction.

The curare-like activity is present only in sera containing antibodies against the cholinoceptors. This activity does not depend on the presence of complement and is unlikely to be due to non-specific effects on the *in vitro* preparations since only neuromuscular transmission is affected. Furthermore the serum neutralized with the receptor protein is devoid of any activity.

It is interesting to observe that the antibodies are active not only against the electroplax receptors (Patrick, Lindstrom, Culp & McMillan, 1973), but also against the nicotinic receptors of animals of different species. This is further substantiated by the recent reports on the possibility of inducing immune flaccid paralysis in rats and guinea-pigs (Heilbronn, personal communication) and monkeys (Tarrab-Hazdai, Aharonov, Silmann & Fuchs, 1975).

At the present time we do not know the mechanism by which the antibodies inactivate the receptor

protein. Inhibition of receptor activity may result from direct interaction of the antibody-combining sites with the active sites of the receptor molecule or from restriction in the diffusion of the agonist into the active sites by steric hindrance, or from conformational changes of the receptor molecule produced by binding of the antibody. Such mechanisms have been proposed, and partially proved, by studies on the effects of antibodies on enzyme activity (Gurari, Silmani & Fuchs, 1974). Furthermore we do not know if the antibodies as well as altering the ability of receptors to bind acetylcholine molecules also inhibit the receptor control of ionic permeability.

In conclusion the data reported here clearly support the hypothesis that the paralysis that Patrick & Lindstrom (1973) described in rabbits and that we have further examined (Clementi *et al.*, 1975) is brought about by circulating antibodies that inactivate the nicotinic receptors. Our observations could be correlated with the claims of a curarizing substance present in myasthenic sera (Berg, 1953; Nastuck *et al.*, 1959; Walker, 1973; Laurent, 1973) and with a recent finding in these patients of a γ-globulin that inhibits the binding of a specific toxin to cholinoceptors (Alman *et al.*, 1974).

Furthermore from our experiments it appears that the nicotinic receptor proteins present early in phylogenesis still maintain a close antigenic relationship with the nicotinic receptors of mammals. This is not a common phenomenon among some of the proteins at the cholinergic junction. For example, it has been observed that antibodies against choline-acetyltransferase of mammals do not cross react with the enzyme of non-mammalian species (Singh & McGeer, 1974) and antibodies produced against the *Torpedo* synaptic vesicles do not show a cross reaction against mammalian cholinergic synaptic vesicles (Ulmar & Whittaker, 1974).

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