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THE EFFECT OF DISULPHIDE BOND REDUCTION ON CHOLINORECEPTORS IN CULTURED SKELETAL MUSCLE

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Abstract—The effects of the disulphide bond reducing agent, dithiothreitol (DTT) were tested on cultured chick embryonic skeletal muscle. Thirty minutes treatment with 10^{-3} M DTT reduced the depolarization produced by acetylcholine and carbachol, but enhanced the depolarization produced by neostigmine. Hexamethonium was converted from an antagonist to an agonist by DTT treatment. Depolarization by high potassium concentrations was unaffected by DTT. The effects of DTT on carbachol and hexamethonium were completely reversed after exposure to an oxidizing agent. It was concluded that the receptors in cultured skeletal muscle are affected by disulphide bond reduction similarly to adult receptors and that the relationship between the disulphide bond and the normal function of the receptor is formed early in the differentiation of muscle.

TREATMENT of electroplax with the disulphide bond reducing agent, dithiothreitol (DTT), decreases the effect of carbachol and acetylcholine, and converts the response of hexamethonium from that of an antagonist to an agonist. These changes can be reversed by oxidation with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).[1,2] It has been postulated that DTT reduces an S-S group on a protein which is close to the acetylcholine binding site on the receptor, and thus alters the conformation and specificity of the receptor itself.[3] Similar results have been obtained with frog *rectus abdominis*[4] and *sartorius* muscles,[5–7] denervated rat skeletal muscle,[8] chick biventer cervicis muscle,[9] and leech dorsal muscle.[10] Despite species variation,[7,10] DTT does appear to affect the properties of the nicotinic receptor in skeletal muscle.

The purpose of the work described here was to examine the effects of DTT on skeletal muscle grown in cell culture and to compare the behaviour of the receptors present in cultured muscle to that of receptors in adult muscle.

METHODS AND MATERIALS

Cultures of leg muscles from 10–11 day chick embryos were prepared as described in earlier publications.[11,12] The techniques used for electrophysiological recording of membrane potentials and for drug addition have been described in detail.[13]

Experiments were performed at room temperature (20–22°) after exposure of cultures to DTT at 37°. Cultures with mean resting membrane potentials in the range of 20–40 mV (inside negative) were used in this study and, for comparison, depolarization responses were expressed as per cent reduction from control resting potential levels. The percentage depolarization of the membrane of a cultured muscle fibre to

a given concentration of agonist is independent of the resting potential.[13] Measurements of membrane potentials in the presence of agonist were made during the first 5 min of contact, and the values obtained were averaged.

Drugs used were: carbachol (carbamylcholine chloride, Aldrich), dithiothreitol (Calbiochem), hexamethonium bromide (Koch-Light), acetylcholine chloride, neostigmine methylsulphate and 5,5′-dithiobis-(2-nitrobenzoic acid) (all Sigma).

RESULTS

DTT in concentrations up to 10^{-3} M and with contact times up to 60 min had no effect on the resting potential of the fibres nor on the fibrillation of spontaneously active fibres. Incubation of cultures with DTT reduced subsequent depolarization by carbachol (Fig. 1). 10^{-4} M DTT for 10 min had no effect on the carbachol response

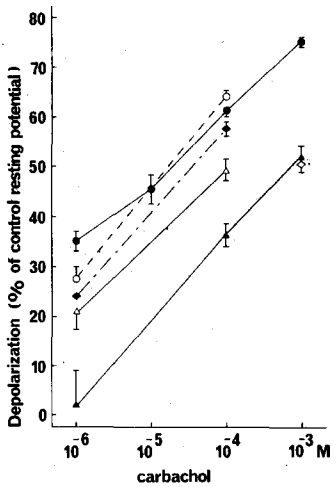

FIG. 1. The effect of DTT treatment on the response to carbachol. Control dose–response curve (●); dose–response curve after 10 min exposure to 10^{-3} M DTT (◆); after 20 min exposure to 10^{-3} M DTT (△); after 30 min exposure to 10^{-3} M DTT (▲); after 60 min exposure to 10^{-3} M DTT (◇); after 30 min exposure to 10^{-3} M DTT, then 10 min exposure to 10^{-3} M DTNB (○). Each point represents the mean ± S.E.M. of experiments on at least 2 cultures including measurements on between 40–150 individual fibres.

but increasing the concentration of the reducing agent to 10^{-3} M produced a significant decrease of the response to 10^{-6} M carbachol, although not to 10^{-4} M carbachol. Increasing the time of exposure to DTT produced a greater reduction in responses to carbachol, the dose–response curve being shifted in a parallel fashion to the right. The maximum effect was obtained after 30 min treatment with 10^{-3} M DTT, and these were, therefore, used as standard conditions in later experiments.

The effects of DTT on the response to carbachol could be completely reversed by 10 min treatment with 10^{-3} M DTNB (Fig. 1). DTNB by itself did not produce depolarization and did not affect the carbachol dose–response curve.

Treatment with DTT resulted in decreased responses to aceylcholine (Fig. 2a) but the depolarization produced by neostigmine[9,13] was enhanced by DTT treatment (Fig. 2c).

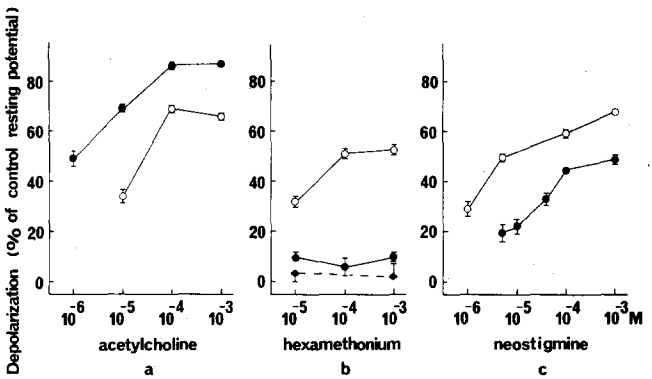

FIG. 2. The effect of DTT treatment on the responses to acetylcholine, hexamethonium and neostigmine. (a) Control dose–response curve to acetylcholine (●); dose–response curve after 30 min exposure to 10^{-3} M DTT (○). (b) Control dose–response curve to hexamethonium (●); after 30 min exposure to 10^{-3}M DTT (○); after 30 min exposure to 10^{-3} M DTT, then 10 min exposure to 10^{-3} M DTNB (◆). (c) Control dose–response curve to neostigmine (●); after 30 min exposure to 10^{-3} M DTT (○).

Hexamethonium was found to be a competitive antagonist at the nicotinic receptors of cultured skeletal muscle, and to have little or no depolarizing activity. However, after incubation of cultures in DTT, hexamethonium acted as an agonist. Addition of 10^{-3} M DTNB for 10 min after DTT treatment eliminated the agonist actions of hexamethonium (Fig. 2b).

The effect of DTT on depolarization induced by high K^+ concentrations was tested. The depolarization produced by 25×10^{-3} M K^+ after DTT treatment was 47·1 ± 1·6% (mean ± S.E.M., n = 84), which was not significantly different from the depolarization produced by a control exposure to the same concentration (44·6 ± 1·6%, n = 176).

The effect of DTT in decreasing responses to acetylcholine and carbachol is similar to the effect of desensitization. If desensitization were due to disulphide bond reduction, the changes observed in the hexamethonium and neostigmine responses after DTT treatment should also be found in desensitized fibres. A conditioning concentration of carbachol was applied.[13] After a short recovery period, test responses to hexamethanium and neostigmine were measured. No depolarization resulted from the addition of hexamethonium, and the depolarization produced by neostigmine was greatly reduced.

DISCUSSION

The results presented in this paper indicate that the receptors on cultured chick embryonic skeletal muscle undergo the characteristic changes in properties following disulphide bond reduction that have been described by earlier workers,[1–10] The changes (decrease in response to carbachol, and conversion of hexamethonium from antagonist to agonist) were as readily reversed by DTNB in cultured muscle as in other systems.[1,2,9] The concentrations of DTT and exposure times required to produce these effects were similar to the conditions employed in other studies.[1,2,4,6–8]

Membrane depolarization by K^+ was not affected by DTT treatment, suggesting that the action of DTT is related to the receptor itself rather than to a nonspecific membrane effect. This is in accord with results of other workers.[1,7,9]

Desensitization has been shown to occur in cultured muscle[13] and it was considered possible that the action of DTT could be equivalent to desensitization. If this was true, desensitization would be expected to increase the depolarizing effects of such compounds as neostigmine and hexamethonium. Since this was not found experimentally it is unlikely that desensitization is the result of disulphide bond reduction.

Our results are consistent with the postulate that the cholinoreceptor is related to a protein containing a disulphide bond susceptible to reduction. The receptors in cultured aneural embryonic muscle and in normal muscle are affected similarly by disulphide bond reduction. The relationship between the disulphide bond and the normal function of the receptor is, thus, apparently formed early in the differentiation of muscle.

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