DECREASING EFFECTS OF GLYCEROL-FRACTIONS EXTRACTED FROM OX DIAPHRAGM MUSCLES ON ACETYLCHOLINE-INDUCED CONTRACTIONS OF SMOOTH MUSCLES

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Abstract—Extracts of 5% glycerol obtained from ox diaphragm muscles were fractionated into four (A, B, C and D) with (NH₄)₂SO₄. The activity as acetylcholine (ACh) receptor-like substance containing fraction was evidenced as follows; the ACh-induced contraction of tracheal muscles decreased with addition of the fraction, and such could not be attributed to the reaction with ACh receptors of tracheal smooth muscles. Fraction D had the most potent activity in the presence of neostigmine. This reaction induced by fraction D was reversed by addition of d-tubocurarine (d-TC). Fraction D was fractionated into three (I, II and III) by gel filtration on Sephadex G-75 with 50 mM phosphate buffer (pH 7.5). The purified fraction III was identified by electrophoresis, UV and visible absorption spectrum, and ion-exchange chromatography to be myoglobin. Pure myoglobin also proved to have a decreasing effect on ACh-induced contraction.

There is a considerable literature concerning isolation of acetylcholine (ACh) receptors from electric organs of electric eels, but little is known of these receptors obtained from mammalian skeletal muscles.

It had been reported that glycerol treatment of rectus abdominis muscles and diaphragm muscles resulted in a gradual loss of contractile activity for ACh (decrease in intrinsic activity) and in the gradual appearance of contractile activity for adenosine 5'-triphosphate (ATP) on denervated diaphragm muscles (1). The pA₂ values for d-tubocurarine (d-TC) of the denervated diaphragms of rats did not change following treatment with 2% glycerol-Tyrode solution (2). These experimental results suggested that the decrease in ACh reactivity induced by glycerol treatment might be partly due to the removal of ACh receptor-like substances from the muscles, and that the substances might be contained in glycerol extracts obtained from diaphragm muscles. It had been reported that glycerol treatment maintained intact the contractile function of muscles even after extraction of about 50% of soluble protein (3). This finding also suggests the possibility of the existence of the ACh receptor-like substances in glycerol extracts.

In the present work, we isolated substances from ox diaphragm muscles and investigated the same for ACh receptor-like properties.

MATERIALS AND METHODS

Extraction and fractionation procedures: Minced diaphragm muscles, from which the
fat of tissue was removed as much as possible before extraction, were extracted with 5% glycerol-Tyrode solution. This concentration of glycerol resulted in an almost total disappearance of ACh-induced contractile responses of denervated diaphragm muscles (2). The procedures of extraction and fractionation were almost the same as already reported (1), and the yields for fraction W, A, B, C and D (crude fraction extracted with 5% glycerol-Tyrode solution and precipitates by 40%, 60%, 80% and 100% saturation with (NH₄)₂SO₄, respectively) were 1.87, 0.28, 0.54, 0.86 and 0.20% in dry weight, respectively. These procedures were performed at a temperature below 4°C. After dialysis with distilled water, each fraction was finally freeze-dried and stored in an ice- stocking.

Fraction D was further fractionated by gel-filtration on Sephadex G-75 (fine particles) with 50 mM sodium phosphate buffer (pH 7.5). To the buffer solution of fraction (0.6 ml), sucrose (25%) was added. The bed dimension of column was 2.0 x 50.0 cm and the flow rate was about 15 ml/hr. Every 3 ml of effluent was collected, and the elution pattern at 280 nm recorded automatically by UVICORD and at 409 nm manually determined by Cary 15.

"Back reaction" activity: The "back reaction" activity, i.e. the decrease of ACh responses induced by these fractions and not caused by antagonistic actions, were assayed by use of isolated rabbit (2.3-4.6 kg weight) tracheal muscles, psoas muscles, male guinea pig (300-450 g) tracheal muscles and frog (28-45 g weight) rectus abdominis muscles immersed in Locke-Ringer's solution at 27°C and in Ringer's solution at room temperature, and bubbled by air, respectively. With respect to crude cholinesterase (ChE) (serum and hemolyzed erythrocyte diluted 3 times with distilled water) prepared from the rabbit, and to each fraction of 5% glycerol extracts, the "back reactions" were assayed. After the contractile responses of tissues induced by ACh (2.2-2.75 μM) reached a plateau, 0.2-1% of each fraction was added to the bath solution. The "back reaction" was reversed by 36.8 μM d-TC administered; d-TC was administered after the "back reaction", or the fraction incubated previously with d-TC for 30 min was administered after the plateau of responses of ACh. This concentration of d-TC did not induce any response by itself. The "back reaction" activity of each fraction to ACh (22 μM) was also studied in the presence of neostigmine (15.0 μM). The effect of fraction W₅ on the contractile responses induced by ATP was studied using glycerinated psoas muscles of rabbit.

Electrophoresis: Electrophoresis of fraction D, I, II and III was performed under the condition of 13.5 V/cm and 5°C for 15 min on agar gel layer (1.5 mm of thickness, 1.5% gel in Tris-EDTA-borate buffer, pH 8.6). Staining with amido black 10 B gave an electrophoretic pattern of the samples.

Materials: Acetylcholine chloride (Daiichi Seiyaku), d-tubocurarine chloride (Nakarai Chemical), adenosine 5'-triphosphate disodium salt (Daiichi Seiyaku) and neostigmine methylsulfate (Merck) were used. Potassium ferricyanide and dithionite was used to identify fraction D as myoglobin (4). DIFCO special agar-noble was used for electrophoresis.
RESULTS

"Back reaction" induced by crude cholinesterase: A basic study of "back reaction" was confirmed first of all prior to the procedure of identification. The contractile responses of rectus abdominis muscles of frogs to ACh (110 μM) were decreased by crude serum ChE (3 mg/ml) as shown in Fig. 1a. This "back reaction" was also obtained by serum, or hemolyzed and diluted blood corpuscle (1 ml). After treatment with neostigmine (15.0 μM) for 1 hr, the contractile responses induced by ACh (0.55-1.1 μM) were not decreased by crude ChE, or crude erythrocyte ChE as shown in Fig. 1b. These results were apparently caused by ChE which hydrolyzed ACh in the bath, and which then decreased the amount of ACh-receptor complex.

"Back reaction" activities of extracted factions (W3, A, B, C and D) in the presence of neostigmine: The "back reaction" activities induced by 0.4% each fraction for rabbit tracheal muscles which were previously contracted by 2.75 μM and 22 μM of ACh in the absence and in the presence of neostigmine (15.0 μM), are shown in Fig. 2. In the absence of neostigmine all tested fractions decreased clearly the responses to ACh, but in the presence of neostigmine the activity of fraction A was greatly decreased, and activities of fractions B and C were almost abolished, whereas activity of fraction D became greater than in the absence of neostigmine, and was most potent in these four fractions. The results suggested that fraction D contained a large amount of substance which reduced ACh-induced contraction of tracheal muscle and which were not ACh-esterase. Fractions A, B and C consisted mainly of ChE and/or non-specific binding fractions for ACh. "Back reaction" induced by the fraction D (2.5-20 mg/ml) increased dose-dependently as shown in Fig. 3. The contractions of 50% glycerol-glycerinated rabbit psoas muscles induced by ATP 6.6 mM in modified Locke-Ringer's solution (pH 7.1) were not inhibited by fraction W3. These "back reactions" were therefore not caused by relaxing factors (5) in these fractions.

Fig. 1. a) The decreasing effect "back reaction" of crude cholinesterase on the frog rectus abdominis muscles contracted by acetylcholine. b) The "back reaction" in the case of preparations treated with neostigmine for 1 hr. Note that the "back reaction" of cholinesterase disappeared with neostigmine, indicating that it depends on the hydrolytic action of cholinesterase.
Comparison of "back reaction" activities induced by W3, A, B, C and D fractions with (blank column) and without (shaded column) 15.0 μM neostigmine. The activity was estimated as the difference in the ACh response before and after administration of each fraction (n-3). Note that the most potent "back reaction" activity with neostigmine occurred in fraction D, indicating that the activity of fraction D is independent on cholinesterase.

Reversal induced by d-tubocurarine of "back reaction": It was necessary to differentiate between a pharmacological antagonistic reaction and the "back reaction". If the fraction contained the pharmacological antagonist for ACh responses of smooth muscles such as tracheal muscles, the reaction should not be interrupted by d-TC. From this point of view, the effects of d-TC on the "back reaction" of fractions W3, A and D were studied as shown in Fig. 4. The "back reaction" of fraction W3 (0.4 and 2.0%) against responses of ACh (2.2 and 11 μM) were reversed by d-TC (71.9 and 287 μM). Similarly the reaction of fraction D (0.5–0.6%) against responses of ACh (2.2–2.75 μM) were reversed by d-TC (36.8 μM).

Reversal of "back reaction": It was necessary to differentiate between a pharmacological antagonistic reaction and the "back reaction". If the fraction contained the pharmacological antagonist for ACh responses of smooth muscles such as tracheal muscles, the reaction should not be interrupted by d-TC. From this point of view, the effects of d-TC on the "back reaction" of fractions W3, A and D were studied as shown in Fig. 4. The "back reaction" of fraction W3 (0.4 and 2.0%) against responses of ACh (2.2 and 11 μM) were reversed by d-TC (71.9 and 287 μM). Similarly the reaction of fraction D (0.5–0.6%) against responses of ACh (2.2–2.75 μM) were reversed by d-TC (36.8 μM).
administered, whereas the reaction of fraction A (0.5%) was not quite reversed by \( \alpha \)-TC. There was moreover a significant difference in the "back reaction" activities of fraction D (1%) against 2.2 \( \mu \)M ACh in the presence and absence of 14.7 \( \mu \)M \( \alpha \)-TC by \( t \)-test; \( t_0 = 8.84 > t_7 = 2.45 \) at a significant level (\( p = 0.05 \)). The reversal caused by \( \alpha \)-TC of "back reaction" was dependent on the concentration of \( \alpha \)-TC (7.4-29.4 \( \mu \)M) as shown in Fig. 5. The above results suggest that fraction D contained ACh-binding substances which are not pharmacologically antagonistic against ACh.

**Further fractionation and identification:** Fraction D was further fractionated by gel filtration on Sephadex G-75 with 50 mM phosphate buffer (pH 7.5). 0.6 ml fraction D (19 mg protein/ml) were admitted to the column, and the elution pattern was represented as optical density at 280 nm and 409 nm in Fig. 6. Three peaks of fractions I, II and III were obtained in 1.6, 0.7 and 97.7% yields respectively. These three fractions were also confirmed by agar gel electrophoresis in Fig. 7. Fraction D was observed to be mainly composed of fraction III. Fraction D in 50 mM phosphate buffer (pH 6.6) was observed to have a characteristic absorption maxima at about 280, 409, 500 and 630 nm. This pattern seemed to correspond to that of myoglobin (4). When fraction D was fractionated by ion-exchange

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**Fig. 5.** Dose-dependent reversal of "back reaction" activity of fraction D induced by 7.4, 14.7 and 29.4 \( \mu \)M \( \alpha \)-tubocurarine in rabbit tracheal muscles.

**Fig. 6.** Effluent diagram of fraction D in O.D. 280 nm ( ) and O.D. 409 nm ( ) added to Sephadex G-75 with 50 mM phosphate buffer pH 7.5. Note three peaks in the O.D. 280 nm.
chromatography on DEAE-cellulose with Tris-HCl buffer (pH 8.4) with a step-wise change in the ionic strength (50 and 5 mM Tris-HCl buffer pH 8.4) (6), visible absorption spectra of the fraction eluted at first was observed to accord with that of ferriyoglobin, and that of next fraction with that of oxymyoglobin. Under this condition, hemoglobin was not eluted. On the other hand, fraction D was transformed into its met-type by potassium ferricyanide, and into its oxy-type by dithionite (4). Visible absorption spectra of these two types of fraction III supported the above suggestion.

Myoglobin which was extracted from ox heart by another method (4, 6) had almost the same degree of “back reaction” activity as fraction D, as shown in Fig. 8.

**DISCUSSION**

The external addition of crude ACh-esterase to the bath decreased the contractile responses of frog rectus abdominis muscles induced by ACh, but did not decrease responses in the presence of neostigmine. However, addition of fraction D from ox diaphragm decreased the contractile response induced by ACh in the presence of neostigmine. This phenomenon seemed to be due to the dissociation reaction of ACh-receptor complexes which we termed “back reaction”. The possible reaction mechanisms were as follows;
where the contraction of muscle segments induced by ACh (A) is dependent on the interaction (AR) of ACh molecule with ACh receptor (R). The contraction is inhibited by the administration of a fraction (R') which binds ACh, "back reaction": (A) + (R') \rightarrow (AR'), and this "back reaction" is reversed by the competitive antagonist of ACh, d-TC (B). It is possible, therefore, that the "back reaction" is available for a bioassay of ACh-binding activity to identify ACh receptor-like substances.

Experimental results with respect to the "back reaction" activity in the presence of neostigmine suggested the possibility that fraction D might contain ACh-binding substances. The facts that fraction III contained mainly myoglobin, which also had the "back reaction" activity, suggested some relation of the substances to ACh receptor mechanisms. As myoglobin is not present in the plasma membrane, such can be ruled out regarding an ACh receptor junction. These receptors are electrophysiologically regarded to be just at the cell surface (7). Amako et al. reported that myoglobin could be extruded from muscles by crush, ischemia, anemia and denervation (8). Myoglobin was considered to move from cytoplasm to the plasma membrane in some cases. The works of Mohr et al. (9) on the interaction between imidazole and hemoglobin as a drug receptor interaction model may support our experimental results.

Fractions I and II further fractionated from fraction D showed much a less yield, and their "back reactions" were not clearly confirmed. Fractions I and II were considered to be proteins of about 50,000 and 20,000-50,000 molecular weight, respectively from the experimental results of the elution pattern as shown in Fig. 8. We speculate that fractions I and II might be also ACh receptor-like substances.

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