Biochemical Pharmacology, Vol. 29, pp. 397–403.
© Pergamon Press Ltd. 1980. Printed in Great Britain.
0006-2952/80/0201-0397 $02.00/0

INFLUENCE OF DECAMETHONIUM AND SUXAMETHONIUM ON THE CONFORMATION OF TRYPTOPHAN SIDE CHAIN CHROMOPHORES OF MEMBRANE BOUND EXTRAJUNCTIONAL ACETYLCHOLINE RECEPTORS

J. PREUNER and T. RÜTHER
Department of Pharmacology, University of Kiel, Hospitalstr. 4-6, D-2300 Kiel, W. Germany

(*Received* 10 *April* 1979; *accepted* 27 *August* 1979)

Abstract—The influence of decamethonium and suxamethonium on the conformation of skeletal plasma membrane proteins was investigated by means of circular dichroism measurements. The CD-spectra of native membranes from both innervated and denervated rat diaphragm resembled the characteristics of proteins containing substantial quantities of α-helix. The development of extrajunctional receptors after chronic denervation induced a change in the protein pattern of the plasma membrane; however, no difference in the overall conformation of the proteins could be detected compared to those of the innervated membrane. It is, therefore, suggested that the newly formed membrane bound acetylcholine receptor protein contains a particularly high content of ordered secondary structure in the same order of magnitude as the other proteins present in the membrane. Decamethonium and suxamethonium gave rise to a distinct change of the CD-spectra of plasmalemmal microsomes derived from denervated rat diaphragm: the occurrence of three additionally ellipticity bands at 235, 215 and near 192 nm. Position and sign of the bands coincided with those known for trytophanyl-*N*-acetylamide. The presence of this side chain chromophore was confirmed by measurements of the trytophan fluorescence of the membrane. The tryptophan fluorescence increased considerably with the time of denervation reflecting a rise in the tryptophan content of the denervated membrane by 55 per cent. Both the increase in tryptophan content and the effect of the neuromuscular agents on the circular dichroism spectra were related to the presence of extrajunctional acetylcholine receptors. No change in protein conformation of the innervated membranes could be detected. Furthermore, this effect could be exerted only when the inside of the microsomes were exposed to the neuromuscular agent during sonication, thus manifesting the inside out nature of the microsomes, i.e. both agents induced a conformational alteration only if they had access to the former extracellular surface of the membrane now facing inwards. Experiments on the concentration dependency of the effect underlined the specifity of the conformational alteration. Suxamethonium induced a conformational alteration in the same concentration range (3×10^{-6}–3×10^{-3} M) as acting upon the intact chronically denervated rat diaphragm. It is suggested that a trytophan side chain chromophore, which is in close vicinity to or part of the acetylcholine receptor, is held in an asymmetric environment, if decamethonium or suxamethonium is bound to the membrane.

Upon binding of cholinergic agonists to isolated nicotinic receptors which are membrane-bound or incorporated into lipid bilayers, changes in the conformation of the receptor protein are induced which mediate changes in the conductance of the Na and K ions [1–6]. The acetylcholine receptor protein is regarded as an integral membrane protein [7–8]. There exists strong evidence that the acetylcholine receptor protein is a transmembrane protein which is exposed at both the extracellular and cytoplasmic faces of the plasmalemma [9]. Underlying alterations of the conformation of the receptor protein, leading to changes in the ion-conductance of the membrane, have been elucidated by measuring changes in the affinity of the receptor binding site for the agonists [10–16]. It was, therefore, of interest to investigate directly whether neuromuscular agents induce conformational alterations of the receptor protein present in the native membrane, leaving the local microenvironment of the binding site [17] intact as far as possible.

One approach to yield direct information on the conformation of membranous proteins is obtained by measuring the circular dichroism spectra of isolated plasmalemmal microsomes [18–19]. Since acetylcholine receptors at the skeletal muscle membrane are restricted to their normal location in the end-plate region, a possible change of their conformation will probably remain undetectable. After denervation, however, extrajunctional acetylcholine receptors are present over the entire length of the muscle fibre surface [20–21]. Under this condition an influence of decamethonium and suxamethonium on the circular dichroism spectra of the skeletal muscle membrane enriched in extrajunctional acetylcholine receptors is described. For the sake of comparison the investigations were extended to microsomes obtained from innervated muscles.

MATERIALS AND METHODS

All experiments were performed on male rats (Sprague–Dawley), weighing approximately 90 g. Under hexobarbital anaesthesia (100–150 mg/kg i.p.), the hemidiaphragm of one side was denervated by cutting the nervus phrenicus at the neck [22]. Twelve to sixteen days after denervation the animals were killed by a blow on the head, the denervated hemidiaphragm was removed and mounted in an organ bath containing tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 0.6 mM $MgCl_2$, 8.9 mM $NaHCO_3$, 0.5 mM NaH_2PO_4 and 5.5 mM sucrose) which was maintained at 37° and aerated with 5% O_2 in CO_2. Mechanical responses induced by suxamethonium were recorded by means of an isotonic recording system. The plasma membrane fraction was prepared after the same period of time after denervation, in which the contralateral innervated hemidiaphragm served as control. Two more preparations of diaphragms of animals not subjected to denervation served as additional controls.

Fractionation of muscle. All operations were carried out in the cold (4°). The hemidiaphragms were excised and placed in 0.25 M sucrose (imidazole buffer 10 mM, pH 7.4). The weight ratio of wet muscle to sucrose solution was 1 : 3. Homogenization was effected twice for 7 sec at 90% full speed in a Virtis '45' homogenizer with a time interval of 30 sec.

Discontinous sucrose density gradient. Vesiculated plasma membrane fragments were obtained following the procedure given by Kidwai *et al.* [23] with modifications according to Lüllmann *et al.* [24]. Homogenate (3 ml) was layered on top of each discontinuous sucrose gradient (0.4/0.6/0.8/1.0 M sucrose, imidazole buffer 10 mM, pH 7.4). No EDTA was added. The samples were centrifuged for 50 min at 12,500 r.p.m. using the swinging bucket rotor SW 27 in the Spinco Model L ultracentrifuge (Beckman Instruments Co.). The microsomes of plasmalemmal origin, mainly floating at the interface between 0.25 and 0.4 M sucrose, were drawn off from the gradient and re-centrifuged to form a pellet at 50,000 r.p.m. for 50 min with a 60 Ti rotor. The pellet was resuspended in 0.25 M sucrose and again centrifuged at 50,000 r.p.m. for 50 min. This washing procedure was repeated in order to obtain a preparation in which the supernatant was almost free of soluble proteins. The microsomal fraction, referred to as '. . .vesiculated plasma membrane fraction. . .' was rehomogenized in sodium phosphate buffer (15 mM, pH 7.4) using a glass–Teflon homogenizer, divided into aliquots containing the protein content necessary for circular dichroism measurement (0.05–0.06 mg/ml) and stored at −20°C. Storage time did not exceed 5 days.

Circular dichroism spectra. Circular dichroism spectra (CD) were recorded at room temperature on a CARY Model 60 spectropolarimeter with a Model 6002 attachment for circular dichroism or on a JASCO Model 40 A. The band width was set to 15 Å. All measurements were made in sodium phosphate buffer (15 mM, pH 7.4) using quartz-cells of 1 mm path length. The Cary Model 60 spectropolarimeter was periodically calibrated in its ORD-mode (optical rotatory dispersion) by analytical grade sucrose. The CD-mode of the Cary Model 6002 attachment and the Jasco Model 40 A were then calibrated according to the calculated ratios of peak molecular ellipticity to peak and trough molecular rotations of aqueous solution of *d*-10-campher sulfonic acid standard [25]. The specific ellipticities $[\Psi]$ in deg cm^2 g^{-2} were calculated from $[\Psi] = \theta/L \times c$ where θ is the observed ellipticity, L denotes the optical path length in dm, and c equals the protein concentration in g cm^{-3}, determined by the Folin–phenol method, using human serum albumin as a standard [26]. Since the average molecular weight of peptide residues present in the plasma membrane of rat diaphragm is not known, the 'mean residue ellipticity' could not be calculated.

Vesicle disruption. Before running the CD-spectra, the microsomes had to be disrupted for reasons discussed in the results. Aliquots (0.5–1.0 ml) of the samples were sonicated at 4°C using the microtip of a Braun sonifier (Model Labsonic 1510). Intensity and time of sonication were 50 W and 2 min, respectively. Conditions were the same as previously applied for guinea pig cardiac plasmalemma [24] which proved to be sufficient to obtain the effects demanded without causing structural damage of the membranes from rat diaphragm. Before each measurement a time lapse of 15 min was necessary to equilibrate the samples to room temperature.

Tryptophan fluorescence. Determination of tryptophan fluorescence was obtained by means of a Zeiss u.v. spectrophotometer (Model PMQ II), equipped with two monochromators for excitement and emission. The samples were excited at 286 nm at a band width of 6 nm. The fluorescence was measured at 340 nm, the emission maximum of tryptophan in globular proteins at a band width of 22 nm. The fluorescence expressed in arbitrary linear units was calibrated by a series of dilution of tryptophan standard in 15 mM sodium phosphate buffer at pH 7.4. The fluorescence intensity of various samples is referred to the fluorescence of serum albumin as a standard in protein determination. Measurement of the tryptophan fluorescence and the protein determination according to Lowry *et al.* [26] was carried out subsequently on each sample. To obtain microsomes of homogenously small size, both the samples of innervated and denervated plasmalemma were subjected to sonication.

RESULTS

The circular dichroism spectra (CD - spectra) of both plasma membrane suspensions obtained from innervated and denervated rat diaphragm resembled the characteristics of proteins containing substantial quantities of α-helix. Compared, however, with entirely solubilized model α-helical polypeptides, the ellipticity bands of the unsonicated samples at 225, 210 and 195 nm (not shown) were shifted towards red and relatively low in amplitude, particularly at 210 nm. These distortions of the suspension curves are mainly due to absorption flattening [27] and light scattering effects [28], i.e. optical artifacts depending on the particulate nature of the membranous system. In order to improve the shape of the CD-spectra by

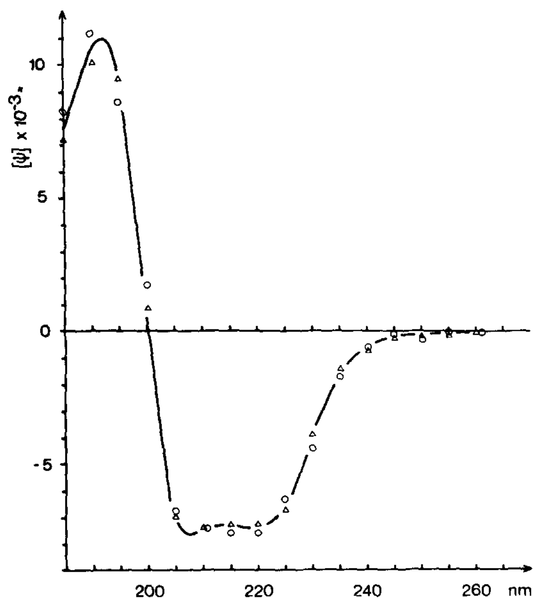

Fig 1. Far u.v. circular dichroism spectra of vesiculated plasma membrane of rat diaphragm. Coinciding specific ellipticities [ψ] of plasma membrane derived from innervated (○—○) and chronically denervated (△—△) rat diaphragm. All samples were sonicated for 2 min at 50 W in order to damp optical artifacts.

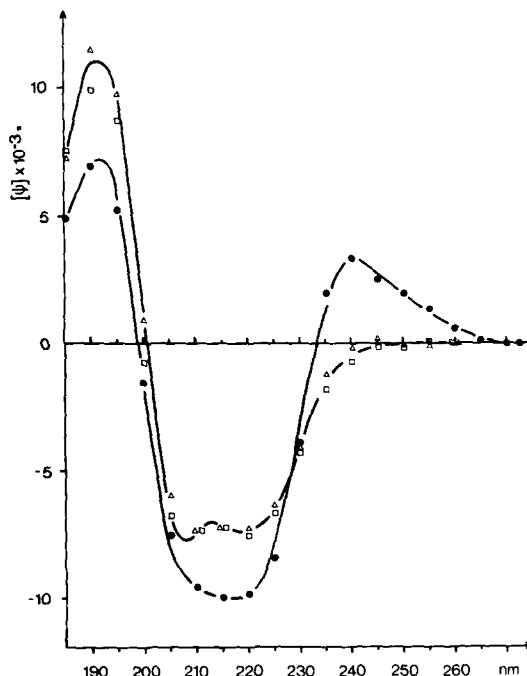

Fig. 2. Effect of decamethonium on the circular dichroism spectra of plasma membranes isolated from chronically denervated rat diaphragm. (●—●) Specific ellipticities [ψ] of vesiculated plasma membrane fragments derived from *chronically denervated diaphragm* sonicated in the presence of 3 × 10^{-4} M decamethonium. (△—△) Control spectrum without addition of decamethonium. (□—□) CD-spectra of plasma membrane derived from *innervated diaphragm*, sonicated in the presence of decamethonium.

decreasing the particle size, the samples were sonicated (see Materials and Methods). After sonication, all bands became more pronounced, particularly at 210 nm, and the spectrum was shifted to lower wave lengths yielding positions of the ellipticity bands at 222, near 210 and at 192 nm (Fig. 1). After deep freezing there was a decrease in the ellipticity of the unsonicated sample, indicating an increase in absorption flattening and light scattering. In freshly prepared membranes, measured prior to deep freezing, the increase of the ellipticity bands on sonication was rather small and detectable only at 210 nm. On sonication, however, the CD-spectra for both the native and frozen plasma membrane suspensions, stored up to 5 days, displayed identical amplitudes and positions of the ellipticity bands. All experiments were, therefore, performed with sonicated samples to ensure comparable conditions.

Denervation of rat diaphragm for a period of 14 days did not change the ellipticity of the vesiculated plasma membrane fraction to a measurable extent. The CD-spectra coincided with those obtained both from control animals and innervated hemidiaphragms of animals which underwent contralateral denervation (Fig. 1).

Experiments carried out on the plasma membrane did not show any conformational changes as a result of decamethonium or suxamethonium up to a concentration as high as 10^{-3} M, when the drug was added either after sonication (Fig. 2) or to non-sonicated membranous vesicles, which were measured in the non-sonicated state. It should be mentioned that the plasma membrane fraction consists of sealed inside-out vesicles [29] which are impermeable for hydrophilic compounds like decamethonium or suxamethonium. After disruption of the vesicles by sonication, the membrane sheets will reseal, yielding homogeneously small vesicles, the membrane of which remains impermeable for the hydrophilic compounds mentioned. It seems, therefore, likely that a lack of change in the CD-pattern might result from inaccessibility of binding sites formerly facing the ECS, now situated at the interior surface of the membrane vesicles. Since the vesicles are only permeable during the time of sonication, the membrane fraction was sonicated in '. . .the presence of decamethonium. . .'. Under this condition a distinct alteration of the CD-spectra was produced by decamethonium which had access to the internal surface of plasma membrane vesicles obtained from chronically denervated diaphragm (Fig. 2). In contrast, the CD-spectra of membrane fractions derived from innervated muscles remained uninfluenced by decamethonium (Fig. 2). Taking into account the inside-out nature of the vesicles, it has to be concluded that decamethonium produced a conformational alteration only via binding sites located at the extracellular surface of chronically denervated rat diaphragm muscle. The same change of the CD-pattern was also detectable in the presence of suxamethonium. *d*-Tubocurarine could not be included in these experiments since the drug itself displayed a strong and overlapping CD-spectrum which made an analysis of the resulting spectra impossible.

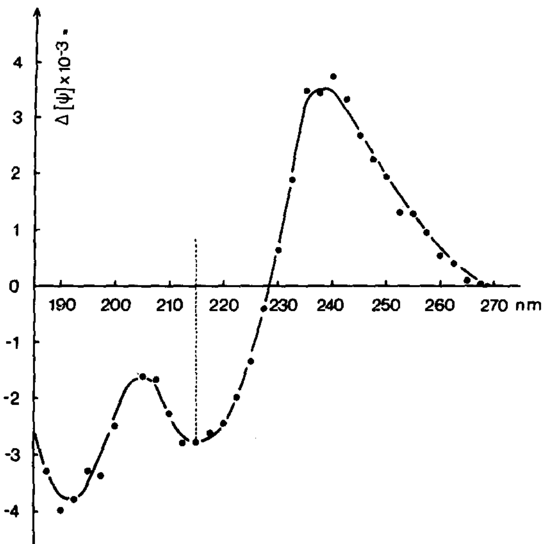

Fig. 3. Difference spectra of the circular dichroism of plasma membranes derived from chronically denervated diaphragm sonicated without and in the presence of decamethonium. Calculated differences of specific ellipticity Δ [ψ] between control spectra (see Fig. 2) and those sonicated in the presence of 3×10^{-4} M decamethonium. Each point represents the average of three different readings. The vertical dotted line indicates the wave length used for the experiments depicted in Fig. 5.

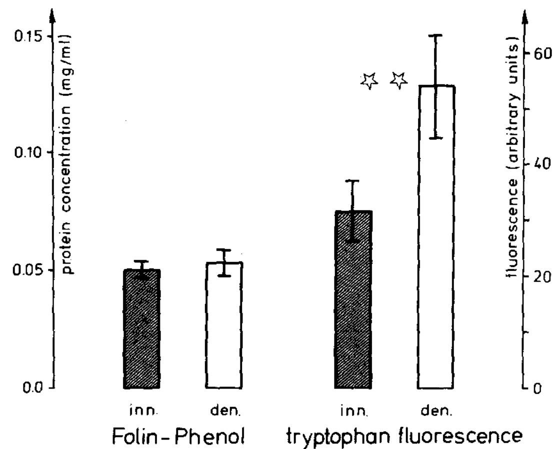

Fig. 4. Tryptophan fluorescence of vesiculated plasma membrane fragments from innervated and chronically denervated rat diaphragm. Left ordinate: protein concentration determined according to the Folin–Phenol method [26]. The protein concentrations of samples from innervated (inn.) and denervated (den.) microsomes used for the determination of the tryptophan fluorescence are identical with those of the CD-spectra (range 0.05–0.06 mg/ml, mean ± S.E., N = 6). Right ordinate: Intrinsic tryptophan fluorescence, given in linear arbitrary units. The samples were sonicated to reduce possible artifacts resulting from differences in particle size between innervated (inn.) and denervated (den.) microsomes. The increase in intrinsic tryptophan fluorescence after denervation is statistically significant (☆☆, $P < 0.01$, mean ± S.E., N = 6).

As shown in Fig. 2, a new positive ellipticity band centered around 235 nm appeared when decamethonium was present during sonication. Simultaneously there seemed to be changes in the region of 230–190 nm. It was difficult to discern whether these changes were additionally evoked and independent from the main bands at 224, 210 and 192 nm or whether they reflected a change in the ratio of the underlying secondary structure of the membrane proteins. In order to evaluate the effects qualitatively, the difference spectrum between the control curve (sonicated vesicles without the drug) and the spectrum in the presence of decamethonium was calculated for each pair of experiments. An example of a difference spectrum thus obtained is depicted in Fig. 3. The resulting difference spectrum confirmed the presence of the positive band at 235 nm. The changes between 230 and 190 nm became apparent as two clearly separable negative bands with maxima at 215 and around 192 nm. Position and sign of the bands allowed them to be distinguished from the three bands commonly present in α-helical proteins, i.e. 224, 210 and 192 nm. Structures which display CD-spectra similar to the difference spectrum observed in the presence of suxamethonium and decamethonium are known for synthetic amino acid derivatives [30]. The *N*-acetylamides of tyrosine, phenylalanine and tryptophan exhibit a positive ellipticity band in the region of 240–220 nm, but they differ in the shorter wave lengths. The CD-spectrum of tryptophanyl-*N*-acetylamide, however, closely resembles the two negative peaks found at 215 and 192 nm in the difference spectrum (Fig. 3). The side chain chromophores of phenylalanine and tyrosine show, on the contrary, strong positive bands in this region.

Consequently, the presence of tryptophan in the membrane was determined by its fluoresence. To ensure comparable conditions with the CD-spectra, the measurements were carried out with sonicated samples at a protein concentration of 0.05–0.06 mg/ml (Fig. 4). Under this condition the instrinsic tryptophan fluorescence of the innervated membrane showed a considerable increase after denervation which amounted to 55 per cent (Fig. 4).

In order to establish a dose–response curve, the effect of suxamethonium on circular dichroism was investigated in concentrations ranging from 3×10^{-6}–3×10^{-3} M. The amplitudes of the difference spectra at 215 nm (see Fig. 3) were plotted vs the concentration of the neuromuscular agent. The threshold concentration of suxamethonium influencing the CD-spectra lay at 3×10^{-6} M. The half maximum effect was exerted at $2–3 \times 10^{-5}$ M, and the maximum effect was reached at 3×10^{-4} M (Fig. 5). The mechanical dose–response curve of the isolated denervated diaphragm stimulated by suxamethonium is included in Fig. 5. The concentration range was similar to that causing the observed changes in the circular dichroism pattern. In the case of decamethonium, a congruence of the two dose–response curves could only be established for the lower concentration range, since overproportional concentrations were required to evoke the maximal mechanical response.

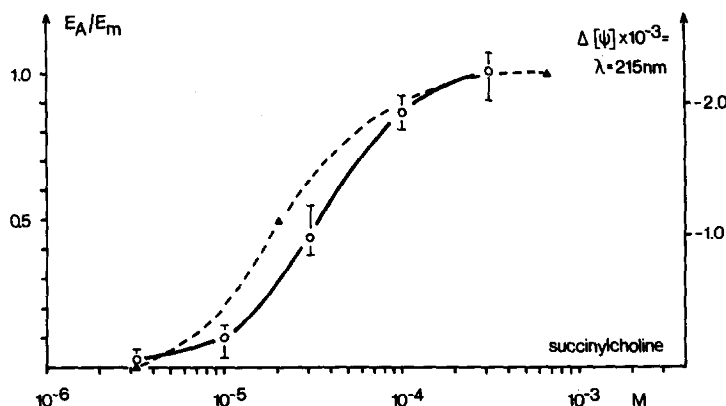

Fig. 5. Dose–response curve of the changes in circular dichroism of plasma membrane derived from chronically denervated rat diaphragm and its relation to the mechanical response induced by suxamethonium at the intact, isolated organ. Abcissa: molar concentration of suxamethonium. Right ordinate: Differences in specific ellipticity ($\Delta[\psi]$) at 215 nm, between the control spectrum and the spectrum obtained in the presence of suxamethonium (○—○) (see Fig. 3). Each point is plotted as the mean of 3–4 readings. Vertical bars represent the range of the experimental data. Left ordinate: Mechanical response (E_A/E_m) (N = 6), (▲—▲). The maximal difference in specific ellipticity at 3×10^{-4} M suxamethonium is set equal to the maximum of the mechanical response possible for the organ ($E_A/E_m = 1.0$).

DISCUSSION

In the present study the circular dichroism spectra of plasmalemmal microsomes have been measured, derived from both innervated and chronically denervated rat diaphragm. In either case identical CD-spectra was obtained (see Fig. 1), although the plasma membrane in the denervated state is enriched in extrajunctional acetylcholine receptors [20–21]. Positions and magnitudes of the ellipticity bands of the skeletal muscles membrane place it into the general category of membranes with a high content of α-helical protein [31]. Since similar values have been reported for the purified receptor protein from *Torpedo nobiliana* [32], it is suggested that both the overall conformation of the membrane and the receptor protein show similar compositions in their secondary structure.

A marked difference was, however, found between innervated and denervated muscle microsomes, when decamethonium or suxamethonium were added to the microsomes and gained access to the intravesicular space by sonication. Whereas the spectrum of the microsomes obtained from innervated muscle remained unchanged, a distinct alteration of the CD-spectra of microsomes obtained from denervated muscle could be observed. It is well established that plasma membrane vesicles from cardiac [33–34] and skeletal [29] muscle consist of inside-out vesicles, the pre-existing structure of which is mainly the transverse tubular system of the plasmalemma. Accordingly, their former extracellular surface is oriented towards the vesicular lumen, the former intracellular surface now facing the incubation medium. The presence of inside-out vesicles, the membrane of which is impermeable for hydrophilic compounds like decamethonium and suxamethonium, demonstrated their sidedness of action. The cholinomimetics investigated produced a conformational alteration of the denervated membrane only via binding sites accessible from the extracellular surface.

The difference spectrum—sonicated microsomes from the denervated diaphragm, with and without the drug—revealed a pattern which closely resembled the CD-spectrum of tryptophanyl-*N*-acetylamide [30]. The similarity suggests that tryptophan might be present as a side chain chromophore which is influenced by the investigated drugs, both agonists of the acetylcholine receptor of the skeletal muscle. The presence of tryptophan in the amino acid composition of purified receptor protein has been reported by several groups [32, 35–37]. If tryptophan does play a role with respect to the alteration of the CD-spectrum induced by cholinomimetic compounds, and increase of the tryptophan content of the plasmalemma should be expected after denervation, since the number of acetylcholine receptors increases after denervation, probably as a result of a *de novo* synthesis of the receptor protein [38–40]. The denervated plasma membrane should, therefore, be composed of a different pattern of proteins compared to the innervated membrane. As shown in Fig. 4, the intrinsic tryptophan fluorescence, expressed in linear arbitrary units, rose by 55 per cent after denervation. This suggests that a specific protein fraction within the total membrane proteins has increased during the time of denervation. It is recognized that the skeletal muscle membrane is more complex than a single protein in solution and that the observed changes in fluorescence could result from a change in protein lipid interactions and/or a specific difference in the conformation between junctional and extrajunctional receptors of the skeletal muscle membrane. Differences have been reported with respect to the rates of turnover

[41–42], the isoelectric point [43] and the ion channel associated with both types of receptor [44–45]. It is still uncertain whether these differences are related to a different type of receptor protein or not [46–49]. In the present experiments, however, the CD-spectra in the absence of neuromuscular agents did not reveal any distinct change in the conformation of membranous proteins between the innervated and denervated state (Fig. 1). It seems, therefore, justified to attribute the increase in fluorescence to an increase in extrajunctional receptor density after denervation.

Specific cholinergic inhibitors like venom α-toxins contain left handed α-helical structure as well as β-structure [50] and, therefore, interfere directly with the CD-spectra of membrane proteins. Also *d*-tubocurarine itself displayed a strong and overlapping CD-spectrum which made the resulting spectra difficult to interprete (see Results). No direct evidence,therefore, can be given whether the tryptophan residue is in close vicinity to or part of the acetylcholine recognition site and its associated ion conductance modulator [48, 51–53]. On the contrary, the dose-dependency of the observed changes of the CD-spectra (Fig. 5) seem to be of particular interest, since the cholinergic ligands exhibit affinities for this component consistent with their pharmacological effectiveness. Suxamethonium induced its effect in the same concentration range as acting upon the intact, isolated denervated muscle.

The experimental data suggest that decamethonium and suxamethonium influence in a dose dependent manner the freedom of movement of a tryptophan side chain chromophore when binding to the acetylcholine receptor from the outer cell surface.

REFERENCES

1. M. C. Goodall, R. J. Bradley, G. Saccomani and W. O. Romine, *Nature, Lond.* **250**, 68 (1974).
2. D. M. Michaelson and M. A. Raftery, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4768 (1974).
3. W. O. Romine, M. C. Goodall, J. Peterson and R. J. Bradley, *Biochim. Biophys. Acta* **367**, 316 (1974).
4. A. E. Shamoo and M. E. Eldefrawi, *J. Membrane Biol.* **25**, 47 (1975).
5. M. E. Eldefrawi, A. T. Eldefrawi and A. E. Schamoo, *Ann. N. Y. Acad. Sci.* **264**, 183 (1975).
6. R. J. Bradley, J. H. Howell, W. O. Romine, G. F. Carl and G. E. Kemp, *Biochem. Biophys. Res. Commun.* **68**, 577 (1976).
7. H. P. Rang, *Q. Rev. Biophys.* **7**, 283 (1974).
8. B. W. Fulpius, *Motor Innervation of Muscle* (Ed. S. Thesleff), pp 1–29. Academic Press, New York (1976).
9. R. Tarrab-Hazdai, B. Geiger, S. Fuchs and A. Amsterdam, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2497 (1978).
10. M. Weber, T. David-Pfeuty and J. P. Changeux, *Proc. natn. Acad. Sci. U.S.A.* **72**, 3443 (1975).
11. D. Colquhoun and H. P. Rang, *Molec. Pharmac.* **12**, 519 (1976).
12. T. Lee, V. Witzemann, M. Schimerlik and M. A. Raftery, *Archs. Biochem. Biophys.* **183**, 57 (1977).
13. G. Weiland, B. Georgia, S. Lappi, C. F. Chignell and P. Taylor, *J. biol. Chem.* **252**, 7648 (1977).
14. N. D. Boyd and J. B. Cohen, *Fedn. Proc.* **37**, 650 (1978).
15. G. Weiland and P. Taylor, *Molec. Pharmac.* **15**, 197 (1979).
16. G. Wieland, D. Frisman and P. Taylor, *Molec. Pharmac.* **15**, 213 (1979).
17. T. Weidmer, U. Brodbeck, P. Zahler and B. W. Fulpius, *Biochim. Biophys. Acta* **506**, 161 (1978).
18. D. W. Urry, *Biochim. Biophys. Acta* **265**, 115 (1972).
19. A. S. Schneider, in *Methods in Enzymology*, Vol. 27, Part D (Eds. C. H. W. Hirs and S. N. Timasheff), pp. 751–767. Academic Press, New York (1973).
20. J. Axelsson and S. Thesleff, *J. Physiol, Lond.* **149**, 178 (1959).
21. R. Miledi, *J. Physiol., Lond.* **151**, 1 (1960).
22. H. Lüllmann and E. Muscholl, *Naunyn-Schmiedebergs Archs. Pharmac.* **221**, 209 (1954).
23. A. M. Kidwai, M. A. Radcliffe, E. Y. Lee and E. E. Daniel, *Biochim. Biophys. Acta* **298**, 593-(1973).
24. H. Lüllmann, T. Peters, J. Preuner and T. Rüther, *Naunyn-Schmiedebergs Archs. Pharmac.* **290**, 1 (1975).
25. J. Y. Cassim and J. T. Yang, *Biochemistry* **8**, 1947 (1969).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. L. N. M. Duysens, *Biochim. Biophys. Acta* **19**, 1 (1956).
28. D. W. Urry and J. R. Krivacic, *Proc. natn. Acad. Sci. U.S.A.* **65**, 845 (1970).
29. A. H. Caswell, Y. H. Lau and J. P. Brunschwig, *Archs. Biochem. Biophys.* **176**, 417 (1976).
30. A. J. Adler, N. J. Greenfield and G. D. Fasman, in *Methods in Enzymology*, Vol. 27, Part D (Eds. C. H. W. Hirs, and S. N. Timasheff), pp. 675–735. Academic Press, New York (1973).
31. G. Saccomani, J. G. Spenney, D. W. Urry and G. Sachs, *J. molec. cell. Cardiol.* **6**, 505 (1974).
32. W. M. Moore, L. A. Holladay, D. Puett and R. N. Brady, *Fedn. Eur. Biochem. Soc. Lett.* **45**, 145 (1974).
33. H. Lüllmann and T. Peters, in *Recent Advances in Studies on Cardiac Structure and Metabolism* (Eds. P. E. Roy and N. S. Dhalla), Vol. 9, pp. 311–328. University Park Press, Baltimore (1976).
34. H. R. Besch, L. R. Jones and A. M. Watanabe, *Circulation Res.* **39**, 568 (1976).
35. M. E. Eldefrawi and A. T. Eldefrawi, *Archs. Biochem. Biophys.* **159**, 362 (1973).
36. J. C. Meunier, R. Sealock, R. Olsen and J. P. Changeux *Eur. J. Biochem.* **45**, 371 (1974).
37. M. E. Eldefrawi, A. T. Eldefrawi and D. B. Wilson, *Biochemistry* **14**, 4304 (1975).
38. D. M. Fambrough, *Science,* **168**, 372 (1970).
39. W. Grampp, J. P. Harris and S. Thesleff, *J. Physiol, Lond.* **221**, 743 (1972).
40. J. P. Brockes and Z. W. Hall, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1368 (1975).
41. P. N. Devroetes and D. M. Fambrough, *J. Cell Biol.* **65**, 335 (1975).
42. J. P. Merlie, J. P. Changeux and F. Gros, *Nature, Lond.* **264**, 74 (1976).
43. J. P. Brockes and Z. W. Hall, *Biochemistry* **14**, 2092 (1975).
44. E. Neher and B. Sakmann, *J. Physiol, Lond.* **258**, 705 (1976).
45. J. O. Dolly, E. X. Albuquerque, J. M. Sarvey, B. Mallick and E. A. Barnard, *Molec. Pharmac.* **13**, 1 (1977).
46. T. H. Chiu, J. O. Dolly and E. A. Barnard, *Biochem. Biophys. Res. Commun.* **51**, 205 (1973).
47. J. O. Dolly and E. A. Barnard, *Fedn. Eur. Biochem. Soc. Lett.* **57**, 267 (1975).
48. J. O. Dolly and E. A. Barnard, *Biochemistry* **16**, 5053 (1977).
49. R. G. Shorr, I. O. Dolly and E. A. Barnard, *Nature, Lond.* **274**, 283 (1978).
50. A. T. Tu, *A. Rev. Biochem.* **42**, 235 (1973).

51. A. T. Eldefrawi, M. E. Eldefrawi, E. X. Albuquerque, A. C. Oliveira, N. Mansour, M. Adler, J. W. Daly, G. B. Brown, W. Burgermeister and B. Witkop, *Proc. natn. Acad. Sci. U.S.A.* **74**, 2172 (1977).
52. M. C. Tsai, N. A. Mansour, A. T. Eldefrawi, M. E. Eldefrawi and E. X. Albuquerque, *Molec. Pharmac.* **14**, 787 (1978).
53. E. K. Krodel, R. A. Beckman and J. B. Cohen, *Molec. Pharmac.* **15**, 294 (1979).