J. Pharm. Pharmacol. 1983, 35: 660–665
Received March 18, 1983

Effect of dithiothreitol on agonist and antagonist actions in frog muscle

TIRZA BLEEHEN, AMANDA L. CLARK AND F. HOBBIGER*

Department of Pharmacology and Therapeutics, The Middlesex Hospital Medical School, Cleveland Street, London W1P 6DB, U.K.

The effects of the disulphide bond reducing agent dithiothreitol (DTT) in the frog rectus abdominis preparation have been investigated. DTT, 1 mM, reduced the potency of the monoquaternary agonists acetylcholine, carbachol and tetramethylammonium and the response to electrical field stimulation; the same applied to nicotine, but the action of edrophonium was unaffected and that of the bisquaternary agonist, decamethonium, was increased. The potency of tubocurarine and gallamine as antagonists was unaltered or slightly reduced by DTT when monoquaternary agonists were used and increased when decamethonium was used as agonist. All these effects of DTT were reversed by the oxidizing agent 5-5′dithio*bis*(2-nitrobenzoic acid) and can be explained by a reduction of a disulphide bond in the vicinity of the anionic site of the nicotinic cholinoceptor. Comparison between these results and published data indicate that there are species differences between nicotinic cholinoceptors at motor endplates. In the guinea-pig ileum preparation DTT reduced the potency of nicotine acting at ganglionic nicotinic cholinoceptors, but had no effect on the agonist response mediated via muscarinic cholinoceptors.

Dithiothreitol (DTT) reduces a disulphide bond in the vicinity of the anionic site of the nicotinic cholinoceptor in the eel electroplax (Karlin & Bartels 1966; Karlin 1969). The conformational change in the receptor produced by this action results in a 3 to 4-fold decrease in the potency of monoquaternary agonists and an increase in the potency of the bisquaternary agonist, decamethonium (C 10). Furthermore, responses to hexamethonium are changed from a conventional antagonist type to an agonist type.

In the chick biventer cervicis muscle preparation, DTT also produces an approximately 4-fold reduction in the potency of monoquaternary agonists (Rang & Ritter 1971), but in the frog rectus abdominis preparation, a 4-fold reduction in potency was reported by Fleisch et al (1974), whereas a 10- to 30-fold reduction in potency was observed by Mittag & Tormay (1970). In the chick biventer cervicis muscle preparation, DTT treatment has no effect on responses to the bisquaternary agonist C10, the potency of tubocurarine as an antagonist is increased 2·4 fold and responses to hexamethonium are converted from an antagonist type to an agonist type (Rang & Ritter 1971). In the dorsal muscle of the leech, following DTT treatment, the potency of carbachol (CCh) is reduced but the type of response to hexamethonium is not changed (Ross & Triggle 1972).

Because of these species differences and in view of the fact that studies on frog muscle constitute an important part of electrophysiological investigations of neuromuscular transmission, we have carried out an investigation of the effects of DTT on the action of agonists and antagonists in the frog rectus abdominis preparation. This paper also includes results of studies of the effects of DTT on agonist action at muscarinic and ganglionic nicotinic cholinoceptors in the guinea-pig ileum preparation.

A preliminary report on part of this work has been published (Bleehen et al 1979).

METHODS

Frog rectus abdominis preparation

The rectus abdominis muscle (species: *Rana temporaria*) was suspended in an organ bath (10 ml capacity) containing frog Ringer solution of the following composition in mM: NaCl, 103; KCl, 1; $CaCl_2$, 0·7; $NaHCO_3$, 3; glucose, 5·6. The pH of the solution, gassed with pure oxygen and maintained at 21 °C, was 8·5.

Following stretching by a 2 g load for 60 min, responses were recorded under isotonic or isometric conditions. For isotonic recording, a resting tension of 200 mg was applied and responses were recorded on a smoked kymograph drum via a Gimbal lever

* Correspondence

with an approximately 10-fold magnification. Isometric responses were recorded under a resting tension of 1 g, via a Grass FT-03C force displacement transducer connected to a Devices M4 polygraph. Agonists were added to the organ bath for 90 s at 5 or 6 min intervals and between drug administrations the preparation was washed 3 times with drug-free Ringer solution.

Field stimulation was applied with silver plate electrodes placed in the organ bath on either side of and close to the muscle. Stimulation (0·5 Hz, supramaximal voltage (20 to 50 V) and 0·25 ms pulse duration) was applied for 20 s at 5 min intervals.

Guinea-pig ileum preparation

Preparations, consisting of 2–3 cm lengths of terminal ileum of adult male guinea-pigs were suspended at 37 °C in an organ bath (10 ml capacity) containing Tyrode solution of the following composition in mM: NaCl, 137; KCl, 2·7; $CaCl_2$, 1·4; $MgCl_2$, 1; $NaHCO_3$, 12; glucose, 5·6. The pH of the solution, gassed with oxygen containing 5% carbon dioxide, was 7·4. Responses of the longitudinal muscle were recorded under a resting tension of 1 g via a frontal writing lever, with an approximately 7-fold magnification and writing on a smoked kymograph drum. Agonists were added to the organ bath for 30 s at 3 min intervals.

Determination of cholinesterase activities

Acetylcholinesterase (acetylcholine acetylhydrolase; E.C. 3.1.1.7) and cholinesterase (acylcholine acylhydrolase; E.C. 3.1.1.8.) activities were determined by the Warburg manometric method at 37 °C, with a gas phase of 95% nitrogen + 5% carbon dioxide. Washed human red cells and human plasma, diluted in 25 mM sodium bicarbonate, were the enzyme sources for acetylcholinesterase and cholinesterase, respectively. Acetylcholine chloride, 10 mM, was used as substrate.

Drugs and chemicals

Agonists: acetylcholine chloride (ACh, Koch-Light), carbaminoylcholine chloride (CCh, Koch-Light), decamethonium iodide (C 10, Koch-Light) edrophonium chloride (Roche Ltd), nicotine hydrogen tartrate (BDH) and tetramethylammonium iodide (TMA, BDH). Antagonists: gallamine triethiodide (May and Baker), hexamethonium bromide (Koch-Light), and tubocurarine chloride (Koch-Light). Dithiothreitol (DTT, Sigma). 5-5′-Dithio*bis* (2-nitrobenzoic acid) (DTNB, Sigma). All solutions were prepared freshly before use.

Evaluation of drug effects

For quantitative assessment of changes in the sensitivity of preparations to agonists produced by either DTT or cholinoceptor antagonists, the dose ratio (DR) was used. $DR = C_2/C_1$, where C_2 is the concentration of agonist which, following exposure of a preparation to DTT or an antagonist, gives the same response as the concentration of agonist, C_1, in the control period. Values used for C_1 and C_2 were always taken from the straight portions of the log concentration-effect curves.

For assessment of antagonist action, each concentration of antagonist was equilibrated with the preparation, usually for 30 min, until a constant effect was seen, and the concentration of antagonist was then increased in a stepwise manner. From a plot of the concentration of antagonist (B) against the dose ratio (DR) minus 1, values of $K = B/DR-1$ were calculated, where K is the concentration of antagonist which reduced the potency of the agonist by 50%.

Statistical analysis

For comparing two groups of data, an unpaired one- or two-tailed Student *t*-test was used. If the probability of the two sample means belonging to the same population was less than 5% (i.e. $P < 0{\cdot}05$), the difference between them was deemed to be statistically significant.

RESULTS

Frog rectus abdominis preparation

Effect of DTT on responses to agonists and electrical stimulation. The addition of ACh to the organ bath produced a concentration-related shortening of the muscle (isotonic recording) or increase in tension (isometric recording) within the same range of concentrations (0·5 to 10 μM). The same applied for CCh (2 to 20 μM) and C 10 (2 to 20 μM).

When DTT (0·25 or 1 mM) was added to the organ bath for periods of up to 120 min, the log concentration-effect curve for ACh was shifted to higher concentrations in a parallel fashion, with no change in the maximum response, and the reduction in potency of ACh was maximal or near maximal after 60 min (Fig. 1). Under these conditions the activity of DTT declined, as shown by the observation that when a bath fluid containing 1 mM was removed from a preparation after 60 min and tested on a new preparation, its effect was less than that of a newly made solution containing 0·25 mM DTT. The effect of DTT on the potency of agonists remained

fairly constant for up to 45 min after removal of DTT from the organ bath and thereafter there was a gradual slow return towards the original sensitivity. The effects of 0·25 and 1 mM DTT were fully reversed by DTNB (1 mM), added to the organ bath for 30 min. In a preparation which had not been treated with DTT, DTNB had no effect on responses to ACh. A higher concentration of DTT (4 mM) reduced the potency of ACh further but also reduced the slope and the maximum of the log concentration-effect curve. This effect could not be fully reversed by DTNB.

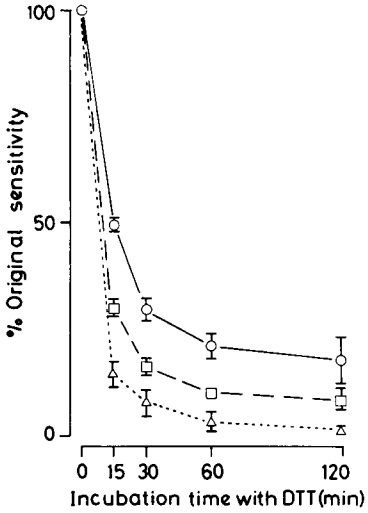

FIG. 1. Effect of DTT on the sensitivity of the frog rectus abdominis preparation to ACh, under isometric recording conditions. Preparations were incubated with DTT (0·25, 1 or 4 mM) for the period stated (abcissa). The sensitivity to ACh is expressed as % of the original sensitivity (ordinate), i.e. as 100/DR %. Figures for DRs following exposure to 4 mM DTT were calculated from concentrations of ACh which produced 50% of the original maximum response. Points and associated bars represent means ± s.e. means (n = 3). ○, 0·25 mM DTT, □, 1 mM DTT, △, 4 mM DTT.

For the quantitative assessment of the effect of DTT on the action of agonists, therefore, a 60 min exposure of the preparation to 1 mM DTT was chosen and agonist responses were obtained within 45 min of removal of DTT from the organ bath.

With the agonists ACh, CCh, TMA and nicotine, DTT (1 mM for 60 min) produced comparable parallel shifts of their log concentration-effect curves to higher concentrations (Fig. 2) which corresponded to an approximately 4-fold decrease in the potency of the agonists. The same result was obtained with isotonic and isometric recording conditions (Table 1).

Responses to edrophonium, in concentrations which exert an ACh-like action, were not significantly affected by DTT (1 mM for 60 min) ($P > 0·05$, Table 1).

Table 1. Effect of DTT (1 mM for 60 min) on responses of the frog rectus abdominis preparation to agonists, under isotonic and isometric recording conditions.

Agonist	Dose Ratios† Isotonic recording*	Isometric recording*
ACh	3·6 ± 0·5 (n = 7)	4·2 ± 0·5 (n = 18)
CCh	3·8 ± 0·7 (n = 7)	3·9 ± 0·3 (n = 27)
TMA	4·1 ± 0·9 (n = 4)	—
Nicotine	—	3·7 ± 0·1 (n = 6)
C 10	0·5 ± 0·1 (n = 7)	0·6 ± 0·1 (n = 7)
Edrophonium	—	1·3 ± 0·2 (n = 3)

† For calculation of dose ratios (DRs), see Methods. Each figure represents the mean ± s.e.m.; n = number of observations.

* For a given recording method, when DRs obtained for ACh, CCh, TMA and nicotine were compared, the differences were not statistically significant ($P > 0·05$), nor was there a significant difference ($P > 0·05$) between DRs obtained for a given agonist when isotonic and isometric recording conditions were compared.

When the bisquaternary compound C 10 was used as the agonist, DTT (1 mM for 60 min) produced a parallel shift of its log concentration-effect curve to lower concentrations (Fig. 2), which represented an approximately 2-fold increase in the potency of C 10. The same result was obtained under isotonic and isometric recording conditions (Table 1).

The effects of DTT on responses to all the agonists tested were fully reversed by DTNB (1 mM for 30 min) (Fig. 2).

Addition of DTT (1 mM) to the organ bath produced a progressive reduction of isometrically-recorded twitch responses of the preparation to field stimulation. This effect reached a maximum within 30 min, at which time the twitch response was reduced to 23·3 ± 9·4% (n=6) of its pre-DTT level. This effect of DTT was comparable to that produced by tubocurarine (0·1 mM) which reduced the twitch response to 21·5 ± 10·0% (n=6) of the control value.

Unlike its effect on agonist responses, the reversibility of the effects of DTNB (1 mM for 30 min) on twitch responses was variable, ranging from 29% to 90% of the control (pre-DTT) twitch tension. The mean reversal was to 61·8 ± 2·4% (n=6) of the control value.

Effect of DTT on the action of antagonists. In preparations not treated with DTT, the log concentration-effect curves for the agonists CCh, ACh, TMA and C 10 were shifted to higher concentrations in a parallel fashion when tubocurarine (0·75 to 15 μM) was added to the organ bath. When the values of (DR-1) for individual agonists were plotted as a function of the concentration of tubocurarine (B), the plots yielded a straight line. Values of

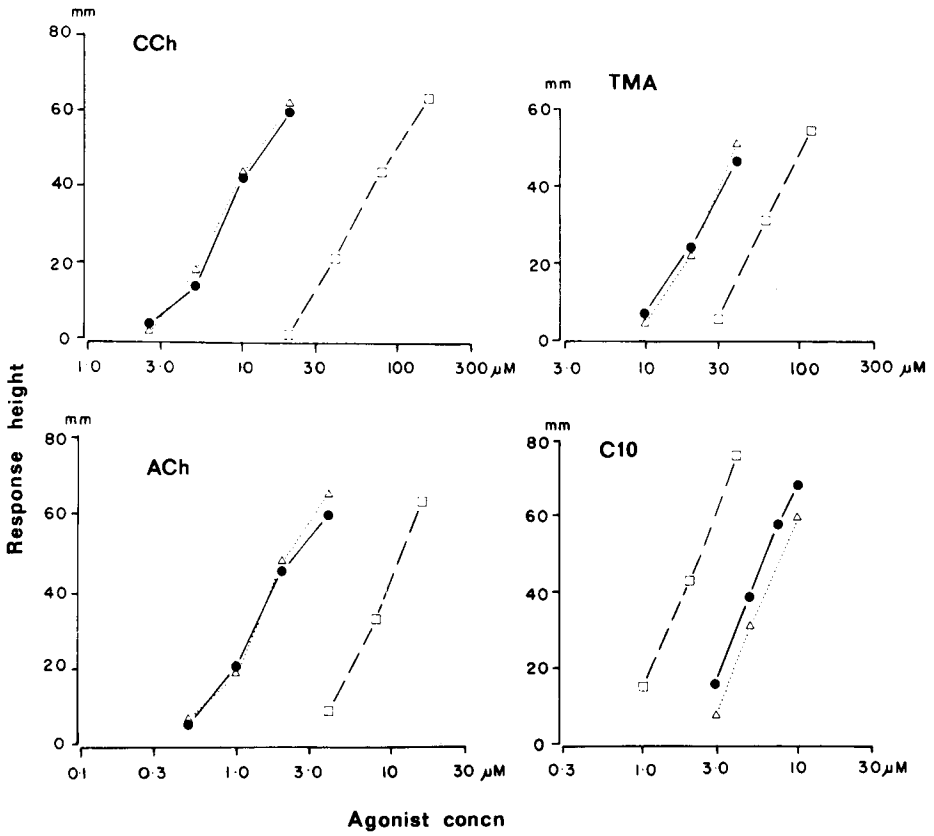

FIG. 2. Effect of DTT (1 mM for 60 min) on responses of the frog rectus abdominis preparation to the agonists CCh, TMA, ACh and C 10, under isotonic recording conditions. The figure shows a typical log concentration-effect curve for each agonist in an individual preparation before (—●—) and after (—□—) treatment with DTT, and after further treatment with DTNB (1 mM) for 30 min (· · · △ · · ·).

K=B/(DR-1) were calculated and are shown in Table 2. There was no significant difference ($P > 0.05$) between K values obtained for an individual agonist when results obtained under isotonic and isometric recording conditions were compared.

Table 2. Effect of tubocurarine on agonist responses of the frog rectus abdominis preparation, with and without DTT treatment; K values†.

	Isotonic recording		Isometric recording	
Agonist	Untreated	DTT-treated	Untreated	DTT-treated
CCh	0·57±0·2 μM (n = 16)	0·60±0·04 μM (n = 16)	0·59±0·06 μM (n = 12)	0·72±0·14 μM (n = 12)
ACh	—	—	0·88±0·11 μM (n = 12)	0·85±0·20 μM (n = 12)
TMA	0·78±0·11 μM (n = 16)	0·84±0·11 μM (n = 12)	—	—
C 10	0·53±0·12 μM (n = 16)	0·31±0·04 μM (n = 20)	0·57±0·06 μM (n = 12)	0·40±0·07 μM (n = 12)

† K = B/DR-1, where B is the concentration of tubocurarine and DR is the dose ratio obtained in its presence. Each figure represents the mean ± s.e.m.; n = number of observations.

* The difference between K values obtained when untreated and DTT-treated preparations were compared, using a given agonist, was statistically significant ($P < 0.001$) only when C 10 was used as the agonist.

For the assessment of the effect of tubocurarine in DTT-treated preparations, account was taken of the slow reversibility of DTT's action on agonist responses. The change in potency of the agonist, which occurred following removal of DTT from the organ bath, was measured as the change in the DR over consecutive 30 min periods and these DRs were used to correct the DRs obtained in the presence of increasing concentrations of tubocurarine over the same periods. Table 2 shows the values of K=B/(DR-1) obtained for DTT-treated preparations. It can be seen that the K values for tubocurarine were not altered by DTT treatment when CCh, ACh and TMA were used as agonists, but K was significantly decreased when C 10 was used as the agonist ($P < 0.001$).

Gallamine (5 to 50 μM) produced parallel shifts of log concentration-effect curves to higher concentrations with the agonists ACh, CCh, and C 10, as assessed under isometric recording conditions. The potency of gallamine was decreased by DTT when

ACh and CCh were used as agonists and increased when C 10 was used as the agonist (Table 3).

Table 3. Effect of gallamine on agonist responses of the frog rectus abdominis preparation, with and without DTT treatment; K values†.

Agonist	Untreated		DTT-treated
CCh	1·78 ± 0·14 μM (n = 14)	*	2·15 ± 0·10 μM (n = 14)
ACh	2·50 ± 0·25 μM (n = 14)	**	4·23 ± 0·61 μM (n = 14)
C 10	2·48 ± 0·27 μM (n = 12)	***	1·34 ± 0·08 μM (n = 12)

† For calculation of K values, see Table 2. Each figure represents the mean ± s.e.m; n = number of observations.

The difference between K values obtained when untreated and DTT-treated preparations were compared was statistically significant using all three agonists (* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$).

Hexamethonium (0·1 to 0·4 mM) produced parallel shifts of log concentration-effect curves for CCh to higher concentrations, as assessed under isotonic recording conditions. The K value for hexamethonium was unaltered by DTT treatment ($P > 0.05$), being 73·6 ± 5·7 μM (n=19) in control preparations and 62·9 ± 7·5 μM (n=15) in DTT-treated preparations.

In 12 out of 15 experiments, following DTT treatment, hexamethonium showed only antagonist type activity. In 3 experiments, hexamethonium (0·4 mM) produced antagonism associated with a small contractile response, amounting to 8 to 13% of the maximum response. The amplitude of the contractile response was not increased by increasing the concentration of hexamethonium.

Guinea-pig ileum preparation

In preparations exposed to DTT (1 mM) the potency of nicotine was reduced. After 30 min incubation with DTT, the DR was 1·3 ± 0·1 (n=4) and after 60 min, it was 3·0 ± 0·5 (n=7). In control experiments, in which 30 and 60 min rest periods were used instead of incubation with DTT, the sensitivity of the preparation increased by factors of 1·7 (DR after rest period = 0·6 ± 0·1, n=4) and 2·5 (DR after rest period = 0·4 ± 0·1, n=4), respectively. Thus, the true DRs produced by DTT were of the order of 2·2 and 7·5 for 30 and 60 min of DTT treatment, respectively.

Incubation with DTT (1 mM) for 30 min resulted in an apparent 2-fold increase in the potency of CCh (DR = 0·5 ± 0·1, n=4). However, a similar increase in potency occurred when the preparation was incubated in the absence of DTT for the same period (DR = 0·5 ± 0·2, n=4). Thus, DTT had no effect on responses to CCh.

Effect of DTT on the activity of acetylcholinesterase and cholinesterase

The addition of DTT, 1 to 10 mM, to acetylcholinesterase caused a progressive reduction of enzyme activity which was prevented by substrate (10 mM ACh) but not reversed by it. The time course of reduction in enzyme activity had first order characteristics and after a 60 min incubation of the enzyme with DTT (under anaerobic conditions) enzyme activity was reduced to 82 ± 3% (n =7) and 23 ± 6% (n = 6) of the control activity, by 1 and 10 mM DTT, respectively.

Cholinesterase activity was less susceptible to the action of DTT and a 60 min incubation of the enzyme with 10 mM DTT reduced enzyme activity only to 87 ± 1% (n = 3) of the control activity.

DISCUSSION

The results of the experiments reported here show that, in the frog rectus abdominis preparation, DTT (0·25 and 1 mM) reduces the potency of the nicotinic agonists ACh, CCh, nicotine and TMA, and increases the potency of C 10, with both effects being fully reversed by the oxidizing agent DTNB. The response to nerve-released ACh is also reduced, which is in agreement with the findings of Ben-Haim et al (1973) and Terrar (1978) that in frog skeletal muscle preparations DTT reduces the postjunctional response to nerve stimulation, i.e. the endplate potential and endplate current.

With higher concentrations of DTT, the effects are more complex since these could not be fully reversed by DTNB. This can explain why Mittag & Tormay (1970) obtained with 2 mM DTT a greater reduction in the potency of ACh than was observed in our experiments and in those of Fleisch et al (1974).

In 1976, Michelson & Shelkovnikov reported that agonists were more potent on the frog rectus abdominis preparation when muscle responses were recorded isotonically than when isometric recording was used. The difference was greatest with the thickest muscles and it was proposed that the muscle shortening in the experiments with isotonic recording, enhanced diffusion. In our experiments all muscles were thin and no differences were observed between the results obtained with isotonic recording and those with isometric recording.

The effects of DTT which we observed in the frog rectus abdominis preparation are comparable to

those seen in the eel electroplax (Karlin & Winnick 1968; Karlin 1969; Silman & Karlin 1969), but differ in some respects from those observed in the chick biventer cervicis muscle preparation (Rang & Ritter 1971). In the latter preparation, DTT reduces the potency of edrophonium, like that of the other monoquaternary agonists, while the response to C 10 is unaffected. Rang & Ritter (1971) also showed that DTT reduced the potency of tubocurarine 2·4-fold, irrespective of the agonist used, whereas in our experiments, the change in potency of tubocurarine and gallamine produced by DTT was dependent on the agonist used, a slight decrease or no change occurring with monoquaternary agonists and an increase occurring when C 10 was used as the agonist. Furthermore, the agonist type response to hexamethonium which consistently occurs in the chick biventer cervicis preparation (Rang & Ritter 1971) following DTT treatment, was only rarely observed in the frog rectus abdominis preparation.

The effects of DTT on agonist and antagonist potency observed in our experiments are consistent with DTT reducing a disulphide bond in the vicinity of the anionic site of the nicotinic cholinoceptor, thereby producing a conformational change in the receptor which alters its affinity for agonists and antagonists (see Karlin 1969). A possible complicating factor, involving an action of DTT on the enzymic hydrolysis of ACh, was ruled out by our findings that under the experimental conditions DTT (1 mM) had only minor effects on the activities of acetylcholinesterase and cholinesterase, as also indicated by the observations of Karlin (1967).

Our results on the guinea-pig ileum preparation show that DTT also effects agonist action on ganglionic nicotinic cholinoceptors, but not on muscarinic cholinoceptors. These findings are in agreement with the observations of Brown & Kwiatowski (1976) using the rat superior cervical ganglion and of Stubbins & Hudgins (1971) and Fleisch et al (1974) using rat jejunum and rat and guinea-pig tracheal preparations, respectively.

It is concluded that, although studies with DTT provide evidence for species differences between nicotinic cholinoceptors of motor endplates, these differences do not exclude the possibility that DTT may be a useful tool in studies of the molecular events associated with inhibition of AChE at mammalian neuromuscular junctions, where muscarinic (Ganguly & Das 1979; Abbs & Joseph 1981) and ganglionic nicotinic (Bowman & Webb 1976; Bowman 1980a, b) cholinoceptors have been implicated in the transmission process.

REFERENCES

Abbs, E. T., Joseph, D. N. (1981) Br. J. Pharmacol. 73: 481–483

Ben-Haim, D., Landau, E. M., Silman, I. (1973) J. Physiol. (London) 234: 305–325

Bleehen, T., Clark, A. L., Hobbiger, F. (1979) Br. J. Pharmacol. 66: 135P

Bowman, W. C. (1980a) Pharmacology of Neuromuscular Function, John Wright and Sons Ltd, Bristol.

Bowman, W. C. (1980b) Anesth. Analg. (Cleve) 59: 935–943

Bowman, W. C., Webb, S. N. (1976) Clin. Exp. Pharmacol. Physiol. 3: 545–555

Brown, D. R., Kwiatowski, D. (1976) Br. J. Pharmacol. 56: 128–130

Fleisch, J. H., Kržan, M. Č., Titus, E. (1974) Am. J. Physiol. 227: 1243–1248

Ganguly, D. K., Das, M. (1979) Nature (London) 278: 645–646

Karlin, A. (1967) Biochim. Biophys. Acta. 139: 358–362

Karlin, A. (1969) J. Gen. Physiol. 54: 245S–264S

Karlin, A., Bartels, E. (1966) Biochim. Biophys. Acta. 126: 525–535

Karlin, A., Winnick, M. (1968) Proc. Natl. Acad. Sci. U.S.A. 60: 668–674

Michelson, M. J., Shelkovnikov, S. A. (1976) Br. J. Pharmacol. 56: 457–467

Mittag, T. W., Tormay, A. (1970) Fed. Proc. Fed. Am. Soc. Exp. Biol. 21: 547 Abs

Rang, H. P., Ritter, J. M. (1971) Mol. Pharmacol. 7: 620–631

Ross, D. H., Triggle, D. J. (1972) Biochem. Pharmacol. 21: 2533–2536

Silman, I., Karlin, A. (1969) Science 164: 1420–1421

Stubbins, J. F., Hudgins, P. M. (1971) Experienta 27: 669–670

Terrar, D. A. (1978) J. Physiol. (London) 276: 403–417