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The relationship between cholinesterase inhibition in the chick biventer cervicis muscle and its sensitivity to exogenous acetylcholine

A. L. GREEN*, J. A. H. LORD† AND I. G. MARSHALL‡

The Department of Biochemistry and the ‡Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, G1 1XW, U.K.

The effect of physostigmine has been studied on cholinesterase in homogenates of chick biventer cervicis muscles and on the contractile responses of the intact muscles to acetylcholine and carbachol. The concentration of physostigmine required to produce the maximum increase in sensitivity to acetylcholine almost completely inhibited the cholinesterase in muscle homogenates. This concentration of physostigmine had no effect on muscle contractures elicited by carbachol. By taking account of the combined effects of acetylcholine diffusion and enzymic hydrolysis, a quantitative theoretical relationship has been derived between the level of cholinesterase activity in cylindrical muscles and the fractional occupancy of the acetylcholine receptors in these muscles in the presence of different concentrations of exogenous acetylcholine. This theory attributes the thousand-fold increase in sensitivity to exogenous acetylcholine produced by anticholinesterases in chick biventer cervicis muscles largely to an alteration in acetylcholine concentration gradient within the muscle and accounts satisfactorily for the shift in the dose-response curve for acetylcholine which occurs after treatment of the muscles with various concentrations of physostigmine.

Anticholinesterase drugs augment responses to exogenous acetylcholine at the neuromuscular junction, but there is as yet no satisfactory quantitative explanation for the great increase in sensitivity to acetylcholine which is frequently observed. The chick biventer cervicis muscle (Ginsborg & Warriner, 1960) is particularly suited to quantitative investigation of acetylcholine potentiation. This muscle is unusually insensitive to acetylcholine in the absence of cholinesterase inhibitors, and such compounds can produce up to a thousand-fold increase in sensitivity to acetylcholine without alteration in the sensitivity to carbachol (Marshall, 1971). Baldwin & Lesser (1971) attempted to correlate the enzyme-inhibiting properties of physostigmine on either intact or homogenized chick biventer cervicis muscles with its augmenting effects on responses to exogenous acetylcholine, but were unable to interpret their results quantitatively. We have re-investigated this problem. It has been shown previously (Green, 1976) that estimations of enzyme inhibition using intact tissues will generally underestimate the fraction of enzyme active centres in the tissues which have been inactivated, and we have used muscle homogenates for all enzyme assays. A new quantitative theory is given, which relates the sensitivity to exogenous acetylcholine to the cholinesterase activity and to the muscle geometry and location of acetylcholine receptors. This theory will account quantitatively both for the thousand-fold increase in sensitivity to acetylcholine produced by physostigmine in the chick biventer cervicis muscle, and for the dependence of the sensitivity on the extent of cholinesterase inhibition.

* Correspondence.
† Present address: Pharmacology Department, Reckitt & Colman Ltd., Hull, U.K.

MATERIALS AND METHODS

Dose-response curves

Biventer cervicis muscle preparations from chicks aged 3–10 days were set up as described by Ginsborg & Warriner (1960), except that dextrose (2 g litre^{-1}) was added to the physiological saline (Krebs & Henseleit, 1932). Muscle contractural responses at 37° were recorded on an ink-writing oscillograph (George Washington 400 MD2) via Grass FT03C force-displacement transducers.

Dose-response curves for acetylcholine and carbachol were obtained by increasing successive doses of the agonists until the respective maximal responses were reached. Each dose of the agonist was left in contact with the tissue for 60 s and was washed out by overflowing the bath for 30 s. After complete relaxation, which took about 1 min, 5 min were allowed to elapse before a further dose of agonist was added. A fresh muscle was used for each complete dose-response curve. Physostigmine or neo-

stigmine was normally added to the tissue bath and left in contact with the preparations for 15 min before adding agonists. With DFP, this pre-incubation period was increased to 30 min. Each log dose-response curve shown in Fig. 2 was constructed from the mean responses (±s.e.) obtained from 4 to 6 separate muscles. Responses were expressed as a percentage of the maximal response to the agonists obtained in the absence of anticholinesterases.

In the absence of an anticholinesterase, the response to acetylcholine reached a plateau in 15 s. In the presence of increasing concentrations of physostigmine the responses to acetylcholine progressively slowed, but even at the highest concentration of physostigmine (3 μM) the response to acetylcholine was close to the plateau level within 60 s (see Fig. 1). The response to carbachol had a time course similar to that of acetylcholine in the presence of high concentrations of physostigmine.

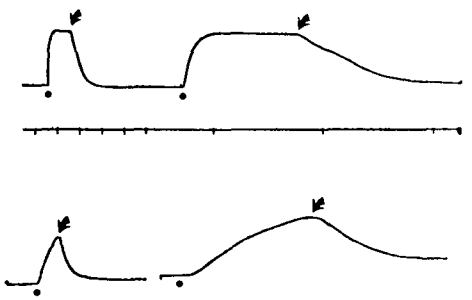

FIG. 1. Chick biventer cervicis muscle preparations. In the upper panel acetylcholine (0·55 mM) was added at ● in the absence of physostigmine. In the lower panel the preparation was treated with physostigmine (3 μM) and acetylcholine (1·7 μM) was added at ●. In each panel the second response was recorded at a higher chart speed. Time markings correspond to intervals of 1 min. At the arrows the tissues were washed by overflow for 30 s.

Cholinesterase assay

Chick biventer cervicis muscles were homogenized for 60 s in ice-cold 0·15 M NaCl, using an Ultra-Turrax TP18/2 homogenizer, to give a final concentration of 100 mg tissue ml^{-1}. Cholinesterase activity was determined by a continuous titration assay method. Acetylcholine bromide (1 ml, usually 0·01 M) was added to a magnetically stirred mixture of homogenate (0·5 or 1 ml), NaCl (0·15 M, 8 or 7·5 ml) and water or physostigmine salicylate (0·5 ml) at 37° and pH 7·4 under a nitrogen atmosphere. The acid produced was continuously titrated to pH 7·4 with NaOH (0·01 M) added from an Agla syringe. The rate of addition was normally between 5 and 25 μl (0·05 and 0·25 μmol) min^{-1}. The physostigmine was normally left in contact with the enzyme for 15 min before adding substrate. Correction for the very slow non-enzymic hydrolysis of acetylcholine was made by running blanks in which the homogenate was replaced by 0·15 M NaCl. All results are the means (± s.e.) of at least three assays.

RESULTS

Effect of anticholinesterases on dose-response curves to acetylcholine and carbachol

Preliminary investigations showed that 15 min pre-incubation of the intact muscles with physostigmine was necessary to obtain the maximum potentiating effect of any concentration of this anticholinesterase.

As shown in Fig. 2, a low concentration of physostigmine (0·03 μM) produced a leftward shift of the log dose-response curve to acetylcholine. At

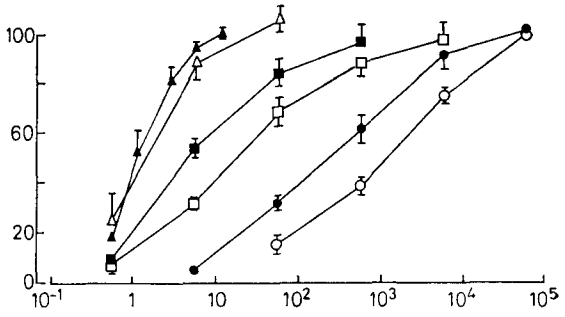

FIG. 2. Dose-response curves for acetylcholine on the chick biventer cervicis muscle after 15 min pre-incubation with physostigmine 0 (○), 0·03 (●), 0·3 (□), 0·6 (■), 1·5 (△) and 3 μM (▲). All results are the mean (± s.e. mean) of 4 to 6 experiments. Ordinate: % of maximal response. Abscissa: Acetylcholine concentration (μM).

0·3 μM there was a greater shift to the left accompanied by an increase in slope. Still higher concentrations (up to 3 μM) produced steadily greater leftward shifts with more marked increases in slope. Concentrations above 3 μM caused no further change in the log dose-reponse curve but occasionally induced contractures in the absence of added acetylcholine. The maximum response to acetylcholine was not significantly altered in the presence of physostigmine.

Sufficiently high concentrations of both DFP (5·5 μM) and neostigmine (3 μM) produced leftward shifts and increases in slope of the acetylcholine dose-response curve almost identical with those produced by 3 μM physostigmine. Physostigmine (up to 3 μM) caused no significant change in the dose-response curve to carbachol.

Cholinesterase activity of homogenates of chick biventer cervicis muscle and inhibition by physostigmine

A Lineweaver-Burk plot showing the dependence of the rate of hydrolysis of acetylcholine by homogenized chick biventer cervicis muscle on substrate concentration at pH 7·4 and 37° is given in Fig. 3. The rate reached a maximum at a substrate concentration of 1 to 2 mM. A concentration of 1 mM was normally used for the inhibition studies. From Fig. 3, the values of Km and Vmax for cholinesterase in this preparation are approximately 100 μM and 50 μmol kg^{-1} s^{-1}. These values are consistent with the observations of Baldwin & Lesser (1971) on the hydrolysis of acetylcholine by chick biventer cervicis muscle homogenate studied manometrically.

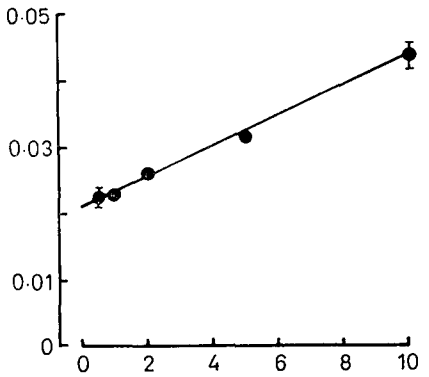

FIG. 3. Lineweaver-Burk plot for acetylcholine hydrolysis at 37° and pH 7·4 by chick biventer cervicis muscle homogenate. All results are the mean (± s.e. mean) of at least 3 assays. Error bars are omitted if smaller than the symbols. Ordinate: 1/v (μmol kg^{-1} s^{-1}). Abscissa: 1/S (mM).

The inhibition produced by physostigmine increased with increasing time of pre-incubation of the enzyme with the inhibitor before addition of substrate, but reached a maximum within 15 min. After this time the inhibition was largely independent of the acetylcholine concentration used in the assay. Similar findings were reported by Stein & Lewis (1969) for inhibition of erythrocyte cholinesterase by physostigmine. After 15 min pre-incubation, physostigmine concentrations of 0·03 and 0·3 μM reduced the cholinesterase activity of the muscle homogenate to 44 ± 4% and 12 ± 2% of controls respectively. A concentration of 3 μM reduced the activity to a level too low for precise measurement (<5% of controls).

THEORY AND DISCUSSION

Under the conditions of our experiments there is strong evidence that the augmentation of responses to acetylcholine by physostigmine is exclusively attributable to inhibition of cholinesterase. Thus the same concentration of physostigmine produced a maximal increase in sensitivity to acetylcholine and almost complete inhibition of cholinesterase, and 15 min pre-incubation with physostigmine was required to produce full effects on both muscle responses and cholinesterase. Additionally, high concentrations of other anticholinesterases produced the same maximum degree of acetylcholine potentiation as did physostigmine. Responses to the agonist carbachol, which is not hydrolysed by cholinesterase, were unaffected by physostigmine.

The response elicited by acetylcholine in any muscle is determined by the local concentration attained at the appropriate receptors on the fibres involved in producing the response. Sub-maximal responses would be augmented if the local concentration rises. Acetylcholine from the bulk medium reaches these receptors by a two stage diffusion process. Firstly, by external diffusion through a stationary layer of medium adjacent to the muscle surface (Cuthbert & Dunant, 1970) and, secondly, by internal diffusion from the surface to the inner fibres. This is illustrated schematically in Fig. 4 for a cross-section of a cylindrical muscle, such as the biventer cervicis. If cholinesterase is present in the muscle a steady state will be reached in which the rate of acetylcholine diffusion across the stationary layer and subsequently to the inner fibres is exactly balanced by the rate of acetylcholine hydrolysis within the muscle. This will create an acetylcholine gradient in which its concentration at the surface is lower than that in the bath, and in which its concentration at the deeper fibres of the muscle will decline steadily from its level at the muscle surface to a minimum at the centre. Only if the cholinesterase is completely inhibited will the acetylcholine at the surface fibres and at the deeper fibres reach the same concentration as that in the medium. Augmentation of responses to exogenous acetylcholine by anticholinesterases could thus result from a rise in the concentration of acetylcholine at surface muscle fibres or from an increase in acetylcholine concentration at the deeper fibres, depending on how much the deeper fibres contribute to the response. It has been shown (Ehrenpreis, 1967; Mittag & Patrick, 1968) that acetylcholine potentiation is not due to preservation of the acetylcholine in the bath. Thus, any quantitative explanation of the extent of acetylcholine

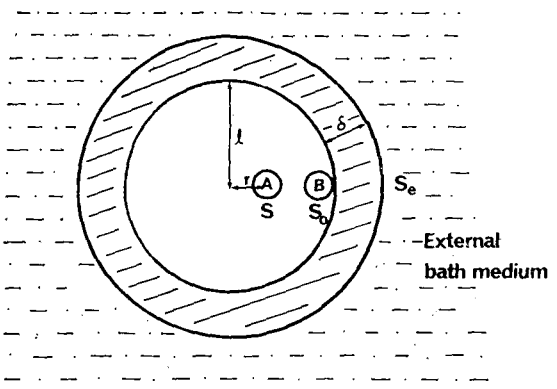

FIG. 4. Schematic representation of cross-section of a chick biventer cervicis muscle. A—inner fibre at distance r from centre: B—surface fibre; hatched area—stationary layer of medium; l—radius of muscle; δ—thickness of stationary layer; S, S_0, S_e—acetylcholine concentrations (see below).

potentiation by anticholinesterases must first require an analysis of the effect of cholinesterase inhibition on the concentration of acetylcholine at the muscle surface, and on the acetylcholine gradient within the muscle.

The concentration of acetylcholine at the muscle surface (S_0)* will reach a steady state relative to the bath concentration (S_e) when the rate of hydrolysis per unit surface area of the muscle (v') exactly equals the rate of diffusion across the stationary layer, that is when $v' = D'(S_e - S_0)/\delta$ where D' is the diffusion coefficient of acetylcholine in the medium and δ is the effective thickness of the stationary layer. For a cylindrical muscle, such as the chick biventer cervicis muscle, the acetylcholine concentration (S) at any point within the muscle bundle at a distance r from the central axis will be related to the surface concentration (S_0) by the equation

$$S = S_0 I_0(\alpha r)/I_0(\alpha l)$$

where I_0 (x) is the modified (hyperbolic) Bessel function, l is the radius of the cylinder and $\alpha = (\text{Vmax}/\text{Km}D)^{1/2}$, where Vmax and Km are the kinetic constants for acetylcholinesterase in the muscle homogenate, and D is the diffusion coefficient for movement of acetylcholine through the muscle (Green, 1976). The diffusion coefficient of acetylcholine in free solution is about 10^{-5} cm^2 s^{-1}(D') but within muscle it is likely to be nearer 2×10^{-6} cm^2 s^{-1} (D) (Krnjevic & Mitchell, 1960). Taking the latter value for D, and the values for Km and Vmax given earlier, the value of α is about 500 cm^{-1}. The radius of the chick biventer cervicis muscle (l) is about 0·04 cm (Baldwin & Lesser, 1971), thus αl is about 20. Provided $\alpha l \gg 1$, it has been shown (Green, 1976) that the rate of hydrolysis per unit volume of muscle (assuming a density of approximately 1 g ml^{-1}) is given by $v = 2\alpha D S_0/l$. Since $v' = v$ multiplied by (volume/surface area) then $v' = \alpha D S_0$, and hence $S_0 = S_e/(1 + \alpha\delta D/D')$. For a number of tissues, the effective thickness of the stationary layer of medium (δ) is about 0·01 cm (Cuthbert & Dunant, 1970). If this value is also applicable to the chick biventer cervicis muscle, the value of $(1 + \alpha\delta D/D')$ is about 2. Thus both S and S_0 may be calculated from S_e in terms of measurable physical and kinetic parameters of the system.

A major problem in all drug-receptor theory is our ignorance of the relationship between receptor occupancy and observed response (Stephenson, 1956). This problem can be partly overcome by assuming that in any particular tissue the same response will always result from the same level of receptor occupancy, and hence the effect of antagonists for example can be interpreted in terms of the change in agonist concentration required to maintain a constant response (pA_x values). This approach can be applied to cholinesterase inhibition in muscles provided all the receptors involved in producing the response are located on the surface fibres of the muscle and hence are all exposed to the same acetylcholine concentration. When the cholin-

* List of symbols

S	acetylcholine concentration at any point within the muscle at a distance r from the central axis.
S_0	acetylcholine concentration at the outer surface of the muscle.
S_e	acetylcholine concentration in the external bath medium.
v'	rate of acetylcholine hydrolysis per unit surface area of the intact muscle.
v	rate of acetylcholine hydrolysis per unit volume of the intact muscle.
Vmax	maximum rate of acetylcholine hydrolysis per unit mass of muscle homogenate.
Km	Michaelis-Menten constant for acetylcholine hydrolysis by the muscle homogenate.
D'	diffusion coefficient for acetylcholine in the medium.
D	diffusion coefficient for acetylcholine in the intact muscle.
l	radius of the muscle.
r	distance of any particular receptor from the central axis of the muscle.
r'	distance from the central axis of the muscle to which acetylcholine must penetrate to elicit a full response.
α	$(\text{Vmax}/\text{Km}D)^{1/2}$.
δ	effective thickness of the stationary layer of medium on the outer muscle surface.
K	dissociation constant for the acetylcholine-receptor complex.
$I_0(x)$	modified (hyperbolic) Bessel function.

esterase is completely inhibited, the surface concentration S_0 becomes the same as the bath concentration S_e. When the cholinesterase is not inhibited, the bath concentration must be raised by a factor $(1 + \alpha\delta D/D')$ in order to maintain the surface concentration at S_0. Thus, if a maximal response of the muscle can be produced by contraction of surface fibres only, cholinesterase inhibition will shift the dose-response curve for acetylcholine to the left in a parallel fashion by a factor of $(1 + \alpha\delta D/D')$, that is, by a factor of two in this case. Even allowing for some uncertainty in the precise values of α, δ, D, and D', this factor is far too small to account for the massive potentiation of acetylcholine by anticholinesterases in the chick biventer cervicis muscle. Occupation of receptors on deeper muscle fibres must therefore be necessary in this instance if acetylcholine is to produce a full response, although this need not be so for all muscles.

If penetration to deep muscle fibres is required for acetylcholine to elicit a maximal response, allowance must be made for the acetylcholine gradient in the muscle which will affect the level of receptor occupancy on different fibres of the muscle in a manner that will depend on the muscle geometry. Since the acetylcholine concentration at the receptors on different fibres will depend on the location of the fibre, the simple approach just described for receptors confined to surface fibres is no longer applicable and a more complex analysis is needed.

If each acetylcholine receptor molecule has only a single acetylcholine binding site, the fractional receptor occupancy at any particular fibre in the muscle is given by $1/(1 + K/S)$, where S is the acetylcholine concentration in the immediate vicinity of that fibre and K is the dissociation constant for the acetylcholine-receptor complex. For a long cylindrical tissue, such as the chick biventer cervicis muscle, the approach of acetylcholine to the receptors on the deeper muscle fibres can be treated purely in terms of the cross-section of the muscle, in other words the only significant motion of the acetylcholine is horizontally through the cross-section from the circumference towards the central axis. Thus, the fractional receptor occupancy at every receptor on a circle in the cross-section at a distance r from the centre is given by

$$1/[1 + K(1 + \alpha\delta D/D')I_0(\alpha l)/S_e I_0(\alpha r)].$$

Provided there are no gross differences in the distribution of receptors on fibres at different levels within the muscle, the net fractional receptor occupancy over the whole muscle can be obtained by integrating the product of this expression and the area of the annulus with a width dr at a distance r from the centre ($2\pi r dr$) over values of r from 0 (or a value r' between 0 and l if only partial penetration of the muscle is required to give sufficient receptor occupancy to elicit a full response) to l, and then dividing by the total area of the cross-section concerned $[\pi(l^2 - r'^2)]$, i.e. net fractional receptor occupancy over the whole muscle =

$$[2/(l^2 - r'^2)] \int_{r'}^{l} \frac{r\,dr}{[1+K(1+\alpha\delta D/D')I_0(\alpha l)/S_e I_0(\alpha r)]}$$

The above expression cannot be integrated in analytical form, but can be evaluated by numerical integration, using a computer program based on Simpson's rule and an algebraic series expansion for I_0, for any value of K/S_e given specified values for α, δ, D, D', l, and r'. Thus the fractional receptor occupancy can be expressed in terms of the bath concentration of acetylcholine (S_e) and the enzymic activity (related to α), as illustrated by the plots in Fig. 5. As can be seen from those curves (the continuous lines) which were drawn assuming that penetration to the deepest muscle fibres was needed for acetylcholine to elicit a full contracture, reduction of α to zero from an initial value of 525 cm^{-1} (i.e. by complete cholinesterase inhibition) would result in a thousand-fold reduction in the concentration of acetylcholine required to cause 50% receptor occupancy. Experimentally, high concentrations of

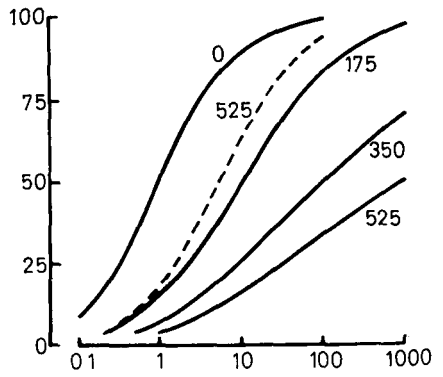

FIG. 5. Theoretical plots of receptor occupancy as a function of normalized acetylcholine concentration (S_e/K) for different values of α (shown against each plot) and a fixed value of l (0·04 cm). The continuous lines assume complete penetration of the tissue by acetylcholine is required to elicit a maximal response. The broken line assumes only 10% penetration of the tissue is required (i.e. r' = 0·036 cm). The values for D, D', and δ are 2×10^{-6} cm^2 s^{-1}, 10^{-5} cm^2 s^{-1} and 0·01 cm. Ordinate: Receptor occupancy (%). Abscissa: S_e/K.

anticholinesterases reduce the concentration of acetylcholine required to cause 50% of a maximal contracture of the chick biventer cervicis muscle by the same amount (from 1·3 mM to 1·3 μM). This value of α is very close to that (500 cm^{-1}) calculated independently for the muscle from the physical and kinetic parameters of the system. Thus, the theoretical model can account quantitatively for the massive potentiation of acetylcholine in this muscle provided it is assumed that occupation of receptors on virtually all the fibres is required in order to elicit a maximum response and that 50% receptor occupancy results in 50% of a maximal response. Cholinesterase inhibition also results in an increase in steepness of the experimental log dose-response curves. A similar increase in slope is observed in the theoretical receptor occupancy-log concentration plots. It can be seen from the broken line in Fig. 5 that if only limited penetration of the muscle were required to elicit a full contracture, the maximum degree of potentiation expected for a given initial enzyme activity would be much reduced and the slope change would be much smaller.

The calculated effect of partial cholinesterase inhibition can also be seen from the plots in Fig. 5. Irreversible and non-competitive cholinesterase inhibitors act by lowering the apparent value of Vmax, reversible competitive inhibitors act by raising the apparent value of Km; either way, the effect is to lower the value of α which shifts the curve to the left and steepens the slope. However, since α is dependent on $(Vmax/Km)^{1/2}$, its numerical value is not related directly to the measured residual enzyme activity in the tissue homogenate, but to its square root. Thus, if the value of α in the absence of physostigmine is 525 cm^{-1}, the values of α corresponding to 56 and 88% inhibition (as produced in the tissue homogenate by 0·03 and 0·3 μM physostigmine) would be 350 and 175 cm^{-1} respectively. Fig. 5 shows that the concentrations of acetylcholine required to produce 50% receptor occupancy at these levels of inhibition are reduced by about 10 and 100-fold respectively. Physostigmine at 0·03 and 0·3 μM reduced the concentration of acetylcholine needed to produce 50% of a maximal contracture by about 6- and 64-fold. The theoretical model can thus also account reasonably quantitatively for the relationship between the extent of acetylcholine potentiation and the level of cholinesterase inhibition.

The above agreement lends strong support to the proposal that in this particular muscle, virtually all the fibres must contract to elicit a maximal response to exogenous acetylcholine and that potentiation by anticholinesterases arises largely from an alteration in the acetylcholine gradient within the muscle. It is probable that similar considerations will apply to other muscles which are very insensitive to acetylcholine in the absence of cholinesterase inhibition and in which anticholinesterases cause massive acetylcholine potentiation, such as the leech dorsal muscle (Flacke & Yeoh, 1968). However, muscles which are very sensitive to acetylcholine in the absence of cholinesterase inhibitors, such as the guinea-pig ileum, may well respond fully to exogenous acetylcholine merely by occupation of receptors on muscle fibres close to the tissue surface (Cuthbert & Dunant, 1970). Augmentation of responses to acetylcholine by anticholinesterases in such instances could still be accounted for as a result of an increase in the surface concentration of acetylcholine, without invoking a requirement for occupation of any receptors on fibres deep within the tissue.

Acknowledgement

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REFERENCES

Baldwin, D. W. & Lesser, E. (1971). *Br. J. Pharmac.*, **42**, 412–422.
Cuthbert, A. W. & Dunant, Y. (1970). *Ibid.*, **40**, 508–521.
Ehrenpreis, S. (1967). *Ann. N.Y. Acad. Sci.*, **144**, 720–734.
Flacke, W. & Yeoh, T. S. (1968). *Br. J. Pharmac. Chemother.*, **33**, 145–153.
Ginsborg, B. L. & Warriner, J. (1960). *Ibid.*, **15**, 410–411.
Green, A. L. (1976). *J. Pharm. Pharmac.*, **28**, 265–274.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyler's Z. physiol. Chem.*, **210**, 33–66.
Krnjevic, K. & Mitchell, J. F. (1960). *J. Physiol., Lond.*, **153**, 562–572.
Marshall, I. G. (1971). *Br. J. Pharmac.*, **42**, 462–472.
Mittag, T. W. & Patrick, P. (1968). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **27**, 472.
Stein, H. H. & Lewis, G. J. (1969). *Biochem. Pharmac.*, **18**, 1679–1684.
Stephenson, R. P. (1956). *Br. J. Pharmac. Chemother.*, **11**, 379–393.