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The effects of neostigmine on the response of the rat anococcygeus muscle to field stimulation are not a consequence of cholinesterase inhibition

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Neostigmine (5×10^{-7} to 5×10^{-6} M) and physostigmine (10^{-5} M) each augment the responses of the rat anococcygeus muscle to field stimulation whereas iso-OMPA (5×10^{-6} to 5×10^{-4} M) or BW 62c47 (5×10^{-7} to 5×10^{-5} M) do not. At a concentration of 10^{-5} M, neostigmine, BW 62c47 and iso-OMPA respectively produced a 48, 50 and 68% inhibition of cholinesterase activity in homogenates of anococcygeus muscle. The ED50 for cholinesterase inhibition by neostigmine ($1{\cdot}4 \times 10^{-5}$ M) is approximately 15 fold greater than the ED50 for the augmentation of the response to field stimulation ($9{\cdot}5 \times 10^{-7}$ M). It is concluded that the action of neostigmine in augmenting the response of the rat anococcygeus muscle to field stimulation is not a consequence of cholinesterase inhibition even though stimulation of muscarinic receptors is implicated.

Smith & Spriggs (1979, 1983) showed that neostigmine altered the shape of the tension response of the rat anococcygeus muscle to field stimulation, causing a shoulder to appear during the relaxation phase. The shoulder was observed at all stimulation frequencies tested between 3 and 40 Hz. In addition at the lower stimulation frequencies (3, 5 and 10 Hz) there was an increase in the maximum peak tension developed. These effects of neostigmine were abolished by low concentrations (5×10^{-8} M) of atropine, but unaffected by (+)-tubocurarine (10^{-6} M).

The mechanism by which neostigmine produced these effects was not determined, although an action on the sympathetic noradrenergic motor nerve terminals was excluded. Histochemical studies using electron microscopy revealed very little cholinesterase staining within the rat anococcygeus muscle and the authors were doubtful that the effects of neostigmine were a consequence of cholinesterase inhibition. The present studies were undertaken to resolve this question.

METHODS

Isolated organ bath preparations

Anococcygeus muscles from male Wistar rats (220–300 g) were dissected out and suspended in Krebs solution (see below) in 3·6 ml organ baths as described by Smith & Spriggs (1983). Tension was measured using ether dynamometer UF1 4 oz isometric transducers and displayed on a Devices M2 pen recorder. Field stimulation (1 ms, 30 Hz, supramaximal voltage for 30 s every 6 min) was applied via parallel platinum wire electrodes mounted in the organ baths.

Assay of cholinesterase in rat anococcygeus muscle homogenates

(a) *Preparation of homogenates*. The preparation of anococcygeus muscle homogenates was adapted from that described by Fonnum (1975). The muscles from 5 rats were pooled, blotted dry and weighed. The 10 muscles, which normally weighed between 100 and 130 mg, were frozen in liquid nitrogen, shattered and the fragments suspended in ice cold 10 mM EDTA buffer at pH 7·4 (10 μl mg^{-1} of tissue, see Drugs and reagents). Triton x-100 (25 μl ml^{-1} of EDTA buffer) was added and the suspension homogenized by hand in an ice-cooled ground glass mini homogenizer for 5 min. The homogenate was centrifuged at 0 °C at 2500 rev min^{-1} for 15 min, and the supernatant drawn off and kept on ice.

(b) *Assay of cholinesterase*. The total cholinesterase activity in homogenates was measured by the colourimetric method of Ellman et al (1961), using acetylthiocholine as substrate. Thiocholine produced by the action of cholinesterase reacts with dithiobisnitrobenzoate present in the reaction mixture to produce a yellow colour. The change in optical density of the reaction mixture at 412 nM was measured at 2 min intervals for 10 min, between 1 and 11 min after starting the reaction. The reaction

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was linear throughout this period. The average change in optical density per minute over the 10 min measurement period was used to calculate enzyme activity.

In the experiments with anticholinesterases, an aliquot of homogenate was preincubated on ice for 30 min with an equal volume of an appropriate concentration of anticholinesterase. The enzyme-inhibitor mixture was then added to the substrate-reagent mixture to start the reaction. All enzyme activity measurements were made at room temperature (22–23 °C).

The enzyme activity in the homogenate decreased slowly with time, usually 10 to 20% over 6 h. To correct for this, control measurements of enzyme activity were made throughout the day. A graph of enzyme activity against time was plotted, from which the enzyme activity at the start of each measurement was interpolated.

Neostigmine and BW 62c47 in solution deteriorated during the course of the day, consequently fresh solutions were made up every 1½ h. Solutions of iso-OMPA were stable throughout the day.

Drugs and reagents

The 10 mM EDTA (ethylenediamine tetraacetic acid, Sigma) buffer with pH 7·4 was prepared as follows. 9·46 g EDTA was dissolved in 20 ml of 0·7 M phosphate buffer pH 7·4, 14 ml of 1 M sodium hydroxide added and the volume made up to 100 ml. To 3·6 ml of this solution was added 14·1 ml of 0·7 M phosphate buffer pH 7·4, and the volume made up to 100 ml.

Drugs used were acetylthiocholine iodide (Sigma), atropine sulphate (Sigma), bethanecol (carbaminoyl-β-methyl choline chloride), dithiobis-nitrobenzoate (Sigma) guanethidine sulphate (Ciba), neostigmine methyl sulphate (Sigma), physostigmine sulphate (BDH), tetraisopropylpyrophosphoramide (iso-OMPA, Sigma), 15-bis-(trimethylammoniumphenyl)-pentan-3-one (BW 62c47, Burroughs Wellcome).

RESULTS

Neostigmine produced a shoulder during the relaxation phase of the response of the rat anococcygeus muscle to field stimulation. Pen recordings of the responses were photocopied and the copied responses cut out and weighed. Shoulder size was quantified by deducting the 'weight' of the control responses from the 'weight' of the response in the presence of neostigmine and is expressed as a percentage of the maximum shoulder weight obtained in that tissue using 2×10^{-5} M neostigmine. The size of the shoulder was linearly related to log concentration in the range 5×10^{-7} M to 5×10^{-6} M neostigmine (Fig. 1). Physostigmine (10^{-5} M) produced a similar shoulder in the response (Fig. 2) which was abolished by atropine (5×10^{-8} to 10^{-7} M). Neither the pseudocholinesterase inhibitor iso-OMPA (5×10^{-6} to 10^{-4} M) nor the true cholinesterase inhibitor BW 62c47 (10^{-7} to 5×10^{-5} M) elicited any modification of the response of the rat anococcygeus muscle to field stimulation.

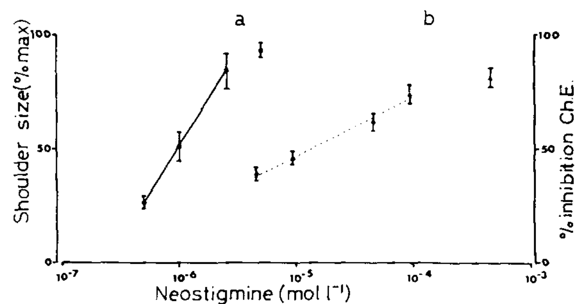

FIG. 1. The relationship between neostigmine concentration and (a) size of the 'shoulder' elicited in the response of the anococcygeus muscle to field stimulation (●, continuous line) and (b) in-vitro inhibition of cholinesterase activity of anococcygeus muscle homogenates (▲, broken line).

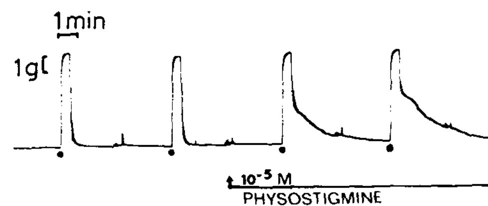

FIG. 2. The effect of physostigmine on the response of the rat anococcygeus muscle to field stimulation. Field stimulation (supramaximal voltage, 30 Hz, 1 ms pulse width for 30 s) was applied at (●). Calibrations, vertical 1 g; horizontal 1 min.

The muscarinic agonist bethanecol contracted the anococcygeus (Fig. 3a), but at sub-threshold concentration ($2·9 \times 10^{-6}$ M) it elicited a large shoulder in the response to field stimulation (Fig. 3b) which was atropine (5×10^{-8} M)-sensitive.

In the presence of high tone induced by guanethidine (10^{-5} M), field stimulation elicited relaxant responses. These relaxations were not appreciably modified by the presence of neostigmine ($2·5 \times 10^{-6}$ M, Fig. 3c).

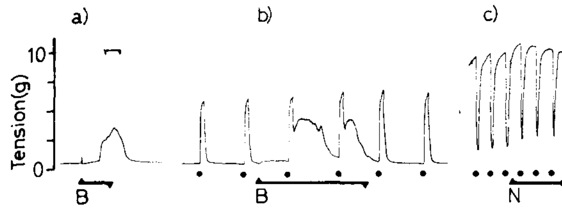

FIG. 3. Responses of the rat anococcygeus muscle to: (a) bethanecol (B), $7 \cdot 3 \times 10^{-6}$ M; (b) field stimulation (●). At B, bethanecol $2 \cdot 9 \times 10^{-6}$ M; (c) field stimulation (●) in the presence of guanethidine ($1 \cdot 16 \times 10^{-5}$ M). At N, neostigmine $2 \cdot 5 \times 10^{-6}$ M. Horizontal calibration 1 min.

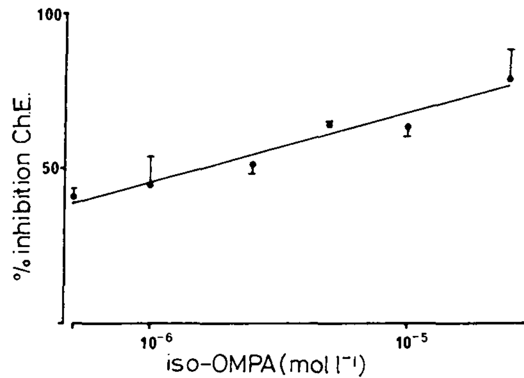

FIG. 4. The in-vitro inhibition by iso-OMPA of cholinesterase activity in homogenates of rat anococcygeus muscle.

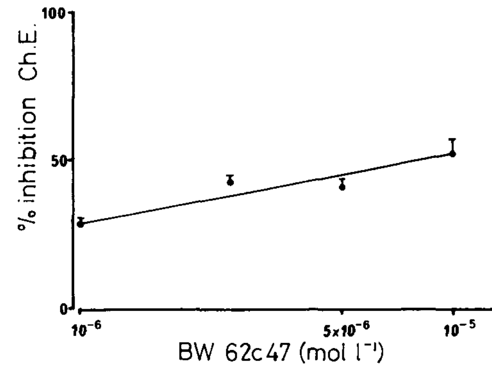

FIG. 5. The in-vitro inhibition by BW 62c47 of cholinesterase activity in homogenates of rat anococcygeus muscle.

Anticholinesterases and cholinesterase activity

Three estimates of total cholinesterase activity from each of three pooled anococcygeus muscle homogenates (10 muscles in each pool), gave a mean value (± s.e.) of $1 \cdot 65 \pm 0 \cdot 13$ μmol min^{-1} g^{-1} tissue. The inhibition of cholinesterase activity by neostigmine was dose related at concentrations between 5×10^{-6} and 10^{-4} M (Fig. 1). The slopes of the log dose-response curves for cholinesterase inhibition by neostigmine and the neostigmine shoulder (Fig. 1) are significantly different ($P < 0 \cdot 001$). The ED50 for cholinesterase inhibition by neostigmine is $1 \cdot 4 \times 10^{-5}$ M, whereas the ED50 for the neostigmine shoulder is $9 \cdot 5 \times 10^{-7}$ M.

Iso-OMPA (5×10^{-7} to $2 \cdot 5 \times 10^{-5}$ M) or BW 62c47 (10^{-6} to 10^{-5} M) produced dose-related inhibitions of rat anococcygeus muscle cholinesterase activity (Figs 4 and 5).

DISCUSSION

Smith & Spriggs (1979, 1983) reported that neostigmine produced an atropine-sensitive, dose-dependent increase in the overall response of the rat anococcygeus muscle to field stimulation. It was considered doubtful that the anticholinesterase was allowing endogenous acetylcholine activity to be enhanced, as electron microscopy histochemical studies revealed little cholinesterase staining in relation to nerve fibres within the body of the muscle, even though dense staining was associated with nerve trunks located on the outer serosal aspects of the muscle.

Physostigmine affects the response to field stimulation similarly to neostigmine and both drugs are inhibitors of both true and pseudo-cholinesterases (Blaschko et al 1949). In contrast, neither the true cholinesterase inhibitor BW 67c47 nor the pseudo-cholinesterase inhibitor iso-OMPA (Diegenbach 1965) elicited changes in the response to field stimulation, despite achieving 50 and 65% enzyme inhibitions respectively at 10^{-5} M. At this concentration neostigmine produced 48% inhibition of enzyme activity and yet elicited its maximum effect on the response to field stimulation. The ED50 for cholinesterase inhibition by neostigmine ($1 \cdot 4 \times 10^{-5}$ M) is nearly 15 fold greater than the ED50 for the increase in response to field stimulation ($9 \cdot 5 \times 10^{-7}$ M), and the slopes of the dose response curves are significantly different. It was concluded from these results that the effect of neostigmine in increasing the response to field stimulation is not a consequence of cholinesterase inhibition.

A criticism which may be levelled at the method used to measure cholinesterase inhibition is that the addition of substrate to the preincubated enzyme-inhibitor mixture may displace the equilibrium resulting in a decrease in inhibition with time. No evidence for this phenomenon was observed in the present experiments as the reaction velocities were

linear over the 10 min measurement period. The rate of change in equilibrium may be too slow to be observed over 10 min or a shift in the equilibrium due to the presence of substrate may be counterbalanced by an opposite shift due to the temperature change in the reaction mixture from preincubation on ice to room temperature.

The mechanism by which neostigmine and physostigmine affect the response of the rat anococcygeus muscle to field stimulation remains unclear. Our results show that inhibition of cholinesterase is not involved, yet Smith & Spriggs (1983) had good evidence that the shoulder resulted from an action at muscarinic receptors, a view supported by the present evidence that sub-contractile concentrations of bethanecol elicit a readily reversible, atropine-sensitive shoulder in the response to field stimulation. The conclusion may be drawn that neostigmine is acting directly at muscarinic receptors. As Smith & Spriggs (1983) showed that the neostigmine shoulder was not due to an action on the noradrenergic motor nerve terminals, there remain two possible sites for these muscarinic receptors. The first is on the smooth muscle itself. Rat anococcygeus muscle possesses motor muscarinic receptors which may receive sub-threshold stimulation from neostigmine which summates with the effects of field stimulation to prolong the response and potentiate sub-maximal responses (see Smith & Spriggs 1983, Fig. 2). The second site is at the sacral inhibitory nerve terminals. Field stimulation results in the release of noradrenaline, and the unknown sacral inhibitory transmitter (Gillespie 1972) and the observed response is thus a combination of the two effects. It is possible that the nerve terminals of the sacral inhibitory nerves possess presynaptic inhibitory muscarinic receptors, such as those demonstrated for sympathetic nerve terminals (Ambache et al 1981). Stimulation of these inhibitory muscarinic receptors by neostigmine would result in field stimulation releasing less inhibitory transmitter, leaving the noradrenergic motor transmitter unopposed. This could result in a stronger and/or more prolonged motor response.

The latter option is less favoured since neostigmine failed to impair relaxant responses to field stimulation in the guanethidine-treated anococcygeus muscle.

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