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Evidence for a Postsynaptic Action of Neostigmine on Muscarinic Receptors in the Anococcygeus Muscle of the Rat

J. A. SMITH AND T. L. B. SPRIGGS

Department of Pharmacology and Therapeutics, University of Wales College of Medicine, Heath Park, Cardiff, UK

Abstract—In the rat anococcygeus muscle neostigmine induces atropine-sensitive, significant leftward displacements of the log concentration-response curves to both noradrenaline and 5-hydroxytryptamine. Responses to high K^+ were also potentiated by neostigmine. However, high K^+ elicited only small and irregular overflows of tritium from [^{3}H]noradrenaline pre-incubated tissues, in contrast to the large overflow elicited by field stimulation. In addition guanethidine blocked responses to field stimulation but not those to high K^+. This is consistent with the dose-related tension responses to high K^+ being elicited postsynaptically. These results indicate that postsynaptic muscarinic receptors are involved in the potentiation by neostigmine of rat anococcygeus muscle responses to field stimulation or exogenous agonists, possibly by an action on receptor operated ion channels. Additional support for a postsynaptic site of action comes from the failure of neostigmine to potentiate tension responses to nerve or field stimulation in the chick expansor secundariorum, a muscle which is devoid of postsynaptic muscarinic receptors.

Neostigmine alters the response of the rat anococcygeus muscle to field stimulation, eliciting a 'shoulder' during the relaxation phase of the response and potentiating the tension developed at lower frequencies of stimulation (Smith & Spriggs 1983). These effects were not attributable to cholinesterase inhibition (Smith & Spriggs 1985) yet were blocked by low concentrations of atropine (5×10^{-8} M) but unaffected by tubocurarine (10^{-6} M). The possibility that neostigmine exerted its effects by stimulating muscarinic receptors was supported by the observation that sub-contractile concentrations of the muscarinic agonist acetyl-β-methylcholine also evoked a shoulder during the relaxation phase of the response to field stimulation (Smith & Spriggs 1985). The present study was undertaken to establish whether these muscarinic receptors were present presynaptically on noradrenergic neurones or post-synaptically on the smooth muscle cells of the anococcygeus muscle.

Materials and Methods

Anococcygeus muscles from male Wistar rats, 220–300 g, were dissected out and suspended in Krebs solution in 3·6 mL organ baths as described previously (Smith & Spriggs 1983). Tension was recorded isometrically, and field stimulation (1 ms, 30 Hz, maximal voltage for 30 s every 6 min) was applied via parallel platinum wire electrodes mounted vertically in the organ baths.

Noradrenaline and 5-hydroxytryptamine (5-HT) log concentration-response curves

After mounting, the anococcygeus muscle was subjected to field stimulation at 6 min intervals until the responses became reproducible (usually 3 or 4 stimulation periods). Field stimulation was then stopped and a concentration-response curve to a single agonist established using a 30 s drug contact time and a 6 min cycle (except with the highest concentrations of drug when the cycle time was extended to allow the tension to return to baseline). The agonist was added in single doses in ascending order of concentration. One tissue from an animal was then used as a control whilst the other tissue was exposed to $2{\cdot}5 \times 10^{-6}$ M neostigmine. Both tissues were stimulated electrically every 6 min until the neostigmine shoulder in the response of the neostigmine-treated (test) tissue became maximal (usually 3 stimulation periods). Responses to 3 concentrations of agonist (which had previously given responses between 20% and 80% of the maximum) were elicited, again in ascending order of concentration. Atropine (5×10^{-8} M) was then added to both baths (in addition to the $2{\cdot}5 \times 10^{-6}$ M neostigmine already in the test tissue bath) and both tissues were subjected to the cycle of electrical stimulation until the neostigmine shoulder in the response of the test tissue was abolished (approx. 3 cycles). Addition of the three agonist concentrations previously selected was again repeated in ascending order of concentration. Responses to agonists were not altered by prior field stimulation nor were responses to field stimulation affected by prior exposure to agonists. Each tissue was exposed to only one agonist.

Potassium chloride concentration-response curves

The K^+ concentrations used were 10, 20, 40, 80 and 160 mM, and were obtained by the addition of 31, 105, 254, 553 and 1149 mg, respectively, of KCl to 100 mL of Krebs solution. Molarities refer to the total K^+ in the Krebs solution after addition of the potassium chloride. Responses to high K^+ solutions were determined using ascending order of concentration and a 10 min cycle with a 30 s contact time. The effects of neostigmine ($2{\cdot}5 \times 10^{-6}$ M) and atropine (5×10^{-8} M) on the responses to K^+ were determined as described above for noradrenaline and 5-hydroxytryptamine (5-HT).

Correspondence to: T. L. B. Spriggs, Department of Pharmacology and Therapeutics, University of Wales College of Medicine, Heath Park, Cardiff, UK.

Statistical analysis

In all experiments where data were subjected to the following statistical analysis, the response of each tissue to a concentration of agonist was expressed as a percentage of the maximum response to the agonist in that tissue. Values are means ± s.e.m. Linear regression lines were calculated for the linear portion of each log concentration-response curve for each tissue. From each regression line the EC50 for the agonist was calculated. The difference between the EC50 values for the first and second log concentration-response curve was calculated for each tissue, a shift of the second curve to the right giving a positive value, and a shift to the left a negative value. The difference in EC50 for the control tissue was subtracted from the difference in EC50 for the test (neostigmine-treated) tissue, to accommodate for variation due to time. The resultant change in EC50 was ascribed to the presence of neostigmine. The pooled results from a series of experiments were tested for significant difference from zero using the t distribution. This procedure was repeated for the first and third, and second and third log concentration-response curves to give the changes in EC50 due to neostigmine plus atropine and atropine alone, respectively.

Guanethidine and responses to K^+

When mounted, the tissues were stimulated alternately for 30 s with 80 mM K^+ or electrical field stimulation using a 10 min cycle. After obtaining control responses the tissues were exposed to 10^{-6} M guanethidine, and 60 min later to 5×10^{-6} M guanethidine, whilst maintaining the stimulation regime.

[^{3}H]Noradrenaline release

Rat anococcygeus muscles were incubated for 20 min with (−)[^{3}H]noradrenaline and washed at 3 min intervals for 45 min as described previously (Smith & Spriggs 1983). Desmethylimipramine (DMI, 10^{-7} M) was present in the Krebs solution during the washout period and throughout the rest of the experiment. When the washing process was complete, the bath fluid was changed every 3 min and a 1 mL aliquot taken for scintillation counting. For one series of 4 experiments the tissues were exposed to a high K^+ concentration for 3 min every fourth sample (12 min). The K^+ concentrations used were 20, 40, 80 and 100 mM, which were obtained by adding potassium chloride to the Krebs (see above). In two other experiments the tissues were exposed to 80 mM K^+ for 10 min, and during the contact period the bath fluid was changed every 2 min instead of every 3 min. In all these experiments at least one period of field stimulation was applied whilst the tissue was bathed in normal Krebs solution (containing 10^{-7} M DMI) to allow comparison between K^+-induced and stimulation-induced release of tritium. Stimulation parameters were 10 Hz, 1 ms, maximal voltage for 15 s. Scintillation counting techniques and the method of quantifying tritium release were all as described previously (Smith & Spriggs 1983).

The chick expansor secundariorum muscle

The expansor secundariorum muscle of domestic fowls (Orchards Farm, Little Kingshill) aged between 6 and 12 weeks were dissected out according to the method of Buckley & Wheater (1968), with the exception that whenever possible the extrinsic nerves were dissected out intact. The preparation was mounted in a 10 mL tissue bath containing Tyrode solution gassed with 5% carbon dioxide in oxygen. Initially the Tyrode solution was maintained at 23°C, but the temperature was raised to 37°C during the course of some experiments. The resting tension of the muscle was maintained at 1 g. Tension was measured isometrically as previously described for the rat anococcygeus (Smith & Spriggs 1983). Field stimulations or exogenous acetylcholine were applied for 30 s every 5 min. Field stimulation of the intrinsic nerves at maximal voltage, 30 Hz, 1 ms, was applied via horizontal platinum wire electrodes (Fig. 1), and the extrinsic nerves were stimulated via bipolar platinum ring electrodes at supramaximal voltage, 0·5 ms, 30 Hz for 30 s.

Materials and reagents

Krebs solution had the following composition (mM): NaCl 94·7, KCl 4·7, $MgSO_4 \cdot 7H_2O$ 1·2, $CaCl_2$ 2·5, KH_2PO_4 1·8, $NaHCO_3$ 25 and glucose 11·7. Tyrode solution had the following composition (mM): NaCl 136·7, KCl 2·7, $MgCl_2$ 1·1, $CaCl_2$ 1·8, NaH_2PO_4 0·4, $NaHCO_3$ 11·9 and glucose 5·6. Stock solutions of (−)-[^{3}H]noradrenaline (Amersham) were prepared by adding the required amount of noradrenaline bitartrate dissolved in deoxygenated distilled water to give 1 Ci $mmol^{-1}$, 180 μCi mL^{-1}, so that 20 μL added to a 3·6 mL organ bath gave a bath concentration of 1 μCi mL^{-1}, 1 μM (−)-[^{3}H]noradrenaline. Drugs used were acetylcholine-*O*-perchlorate (BDH), atropine sulphate (Sigma), 5-desmethylimipramine (DMI, Ciba Geigy), guanethidine sulphate (Ciba Geigy), 5-hydroxytryptamine creatinine sulphate (5-HT, Sigma), neostigmine methyl sulphate (Sigma), noradrenaline bitartrate (Sigma).

Results

Rat anococcygeus muscles

The noradrenaline log concentration-response curve was shifted significantly ($P < 0.001$, n = 6) to the left by 0·79 ± 0·084 log units in the presence of neostigmine (2.5×10^{-6} M), and this was significantly ($P < 0.005$, n = 6)

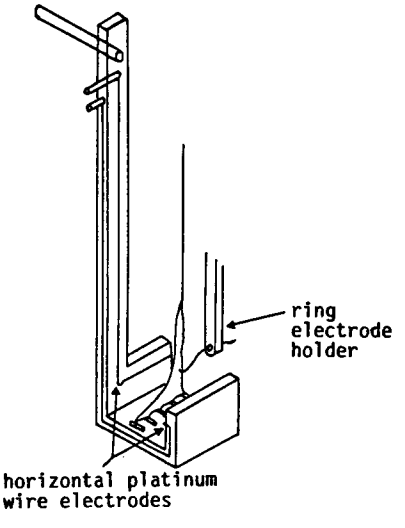

FIG. 1. Combined tissue holder and electrode assembly for nerve trunk and field stimulation of the chick expansor secundariorum muscle.

shifted back to the right by $0{\cdot}7 \pm 0{\cdot}17$ log units following the addition of 5×10^{-8} M atropine. The control noradrenaline log concentration-response curve did not differ significantly ($P > 0{\cdot}3$, n = 6) from the curve obtained in the presence of neostigmine plus atropine.

Similar results were obtained for 5-HT. The 5-HT log concentration-response curve was significantly ($P < 0{\cdot}005$, n = 4) shifted to the left by $0{\cdot}79 \pm 0{\cdot}107$ log units in the presence of $2{\cdot}5 \times 10^{-6}$ M neostigmine, and this was significantly ($P < 0{\cdot}05$, n = 4) shifted back to the right by $0{\cdot}63 \pm 0{\cdot}219$ log units following the addition of 5×10^{-8} M atropine. The control log concentration-response curve to 5-HT did not differ significantly ($P > 0{\cdot}15$, n = 4) from the curve obtained in the presence of neostigmine plus atropine.

In most experiments with noradrenaline and 5-HT the tissues relaxed relatively slowly after exposure to agonist and it was not possible to observe any shoulder in the presence of neostigmine. However, in one experiment neostigmine produced a shoulder in the response to exogenous noradrenaline which was comparable to that produced in the response to field stimulation (Fig. 2). Furthermore there was a small potentiation of this submaximal response to noradrenaline, but no potentiation of the already maximal response to field stimulation (see Smith & Spriggs 1983, Fig. 2). The shoulder in the responses to field stimulation and exogenous noradrenaline, and the potentiation of the noradrenaline response were abolished by atropine (5×10^{-8} M).

The log concentration-response curves to K^+ were not amenable to the same type of statistical analysis. The highest concentration of K^+ used (160 mM) did not produce a maximum response, and the data obtained is insufficient to determine whether the curves are sigmoid in shape. However, in 4 out of 5 experiments neostigmine ($2{\cdot}5 \times 10^{-6}$ M) potentiated the response to all K^+ concentrations tested, and this was reversed at least partially by atropine (5×10^{-8} M) (Figs 3, 4).

In 4 experiments, 60 min exposure to 10^{-6} M guanethidine inhibited responses to field stimulation by $41{\cdot}5 \pm 8{\cdot}2\%$, but only inhibited responses to 80 mM K^+ by $8{\cdot}75 \pm 1{\cdot}6\%$. This difference is significant ($P < 0{\cdot}01$, n = 4). Guanethidine at

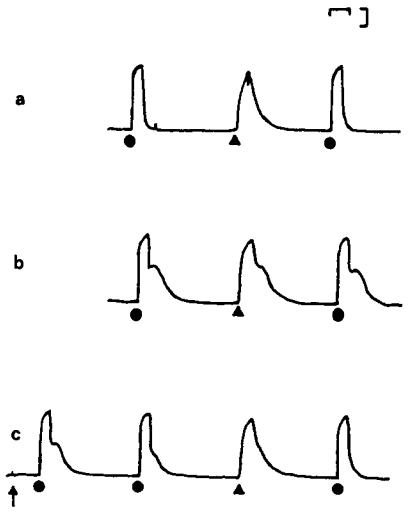

FIG. 2. The effect of neostigmine on the response of the rat anococcygeus muscle to field stimulation (●) or exogenous noradrenaline (▲, $15{\cdot}6 \times 10^{-6}$ M). (a) Control responses. (b) Responses in the presence of $2{\cdot}5 \times 10^{-6}$ M neostigmine. (c) At the arrow, 5×10^{-8} M atropine was added in the continued presence of neostigmine ($2{\cdot}5 \times 10^{-6}$ M). Calibrations; horizontal 1 min; vertical 1 g.

5×10^{-6} M increased the tone in two of the tissues and the inhibition of motor responses by guanethidine could no longer be quantified. In the other two tissues responses to field stimulation were inhibited by 95 and 96%, whereas responses to 80 mM K^+ were inhibited only by 11 and 8%.

Release of tritium by high K^+ with both 3 min and 10 min contact times in the presence of 10^{-7} M DMI was unpredictable and not concentration-related (Fig. 5). Fractional release produced by K^+ was small by comparison with the fractional release induced by field stimulation (Fig. 5). However, K^+ did produce concentration-related contractions of the muscle.

Chick expansor secundariorum muscle

Neostigmine ($2{\cdot}5 \times 10^{-6}$ and 10^{-4}M) had no effect on re-

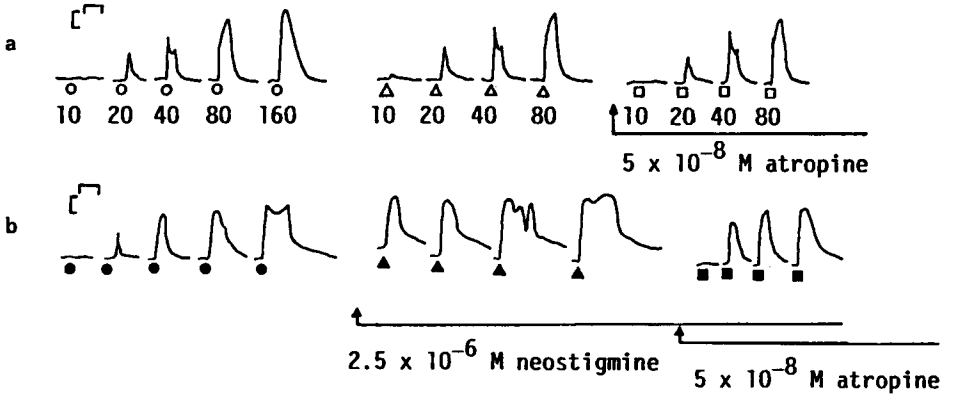

FIG. 3. The effect of neostigmine on the response of the rat anococcygeus muscle to high concentrations of K^+. High K^+ Krebs solutions were added at the symbols, the mM concentration of K^+ being shown underneath each panel in trace a (control tissue). These concentrations also apply to the corresponding panels in trace b. Circles indicate the first concentration response curve, triangles the second and squares the third, with open symbols used for the control tissue and filled symbols for the test. These responses are presented graphically in Fig. 4, where the same symbols are used. Calibrations; horizontal 1 min; vertical 1 g.

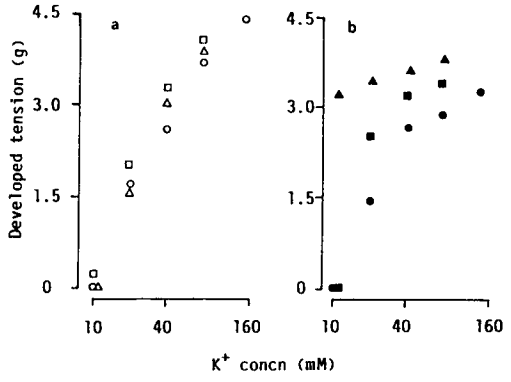

FIG. 4. The effects of neostigmine ($2{\cdot}5 \times 10^{-6}$ M) and atropine (5×10^{-8} M) on the concentration/response curves to K^+ in rat anococcygeus muscle. The responses shown in Fig. 3 are plotted here as tension developed against the log mM concentration of K^+. Graph a corresponds to trace a, and graph b to trace b of Fig. 3 and the symbols correspond to those used in Fig. 3.

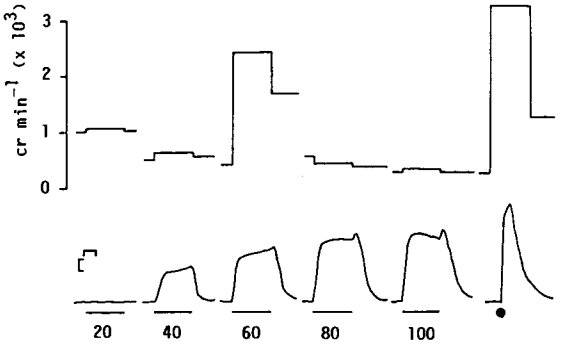

FIG. 5. The effect of high K^+ concentrations on muscle tone and tritium release in the in-vitro rat anococcygeus muscle preincubated with (−)-[^{3}H]noradrenaline. The figure shows typical tension recordings and tritium release from a single experiment. In the lower trace the bar and number below each of the first five panels indicate the presence and mM concentration of K^+ in the bathing solution. In the last panel field stimulation (supramaximal voltage, 30 Hz, 1 ms pulse width for 30 s) was applied at ●. Calibrations; horizontal 1 min; vertical 1 g. The histograms above the panels represent the release of tritium expressed as corrected counts released per minute (cr min^{-1}), for the periods before, during and after exposure to high K^+ or field stimulation.

sponses to field stimulation (9 expts) nor to nerve stimulation (3 expts) either at 23 or 37°C. Acetylcholine in concentrations up to 10^{-5} M had no effect on the muscle, even in the presence of 10^{-4} M neostigmine (4 expts). At 23°C guanethidine produced a partial inhibition of responses to field or nerve stimulation which was slow in onset, but when the temperature was raised to 37°C the inhibition of responses rapidly intensified. At 37°C responses to nerve stimulation were abolished (3 expts), whereas responses to field stimulation were inhibited by 80 to 95% in muscles from 6 to 8 week old chicks (6 expts), and by 60 to 85% in the larger muscles from 10 to 12 week old chicks (4 expts). Fig. 6 illustrates the effects of guanethidine on the responses of the chick expansor secundariorum muscle to nerve or field stimulation at 37°C.

Discussion

A direct stimulant action of neostigmine on muscarinic receptors had been postulated to account for the ability of

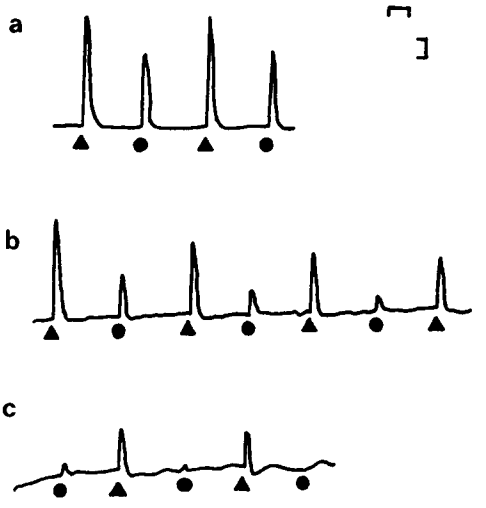

FIG. 6. A continuous record showing the inhibitory effect of guanethidine on responses of the chick expansor secundariorum muscle to nerve or field stimulation. (a) shows control responses to alternate field stimulation (▲, maximal voltage, 30 Hz, 1 ms for 30 s) and nerve stimulation (●, supramaximal voltage, 30 Hz, 0·5 ms for 30 s). (b) shows the record obtained immediately after addition of guanethidine at 5×10^{-6} M and (c) that after guanethidine at 10^{-5} M. Calibrations; horizontal 1 min; vertical 1 g.

neostigmine to potentiate and to elicit a shoulder in the relaxant phase of responses of the anococcygeus muscle to field stimulation (Smith & Spriggs 1983, 1985). From the results of the present study a postsynaptic location for these muscarinic receptors is favoured. Thus neostigmine elicits an atropine-sensitive potentiation of responses to exogenously applied noradrenaline or 5-HT and was shown to elicit a shoulder in the relaxation phase of a response to noradrenaline. The atropine-sensitive, neostigmine-induced potentiation of responses of the anococcygeus muscle to high K^+ provides further support for a postsynaptic site of action. High concentrations of K^+ release neuronal noradrenaline in a number of tissues including rat vas deferens (Bisby & Fillenz 1969), guinea-pig atria (Sorimachi et al 1973) and rat heart (Carpenter & Nash 1976). However, in the present experiments high K^+ elicited only small and inconsistent overflows of tritium from tissues pre-incubated with [^{3}H]noradrenaline. In addition contractions to K^+ were only marginally impaired by concentrations of guanethidine which blocked responses to field stimulation. Consequently the concentration-related contractions of the anococcygeus muscle to K^+ appear to result from a direct action (not receptor mediated) on the smooth muscle cells rather than indirectly by releasing noradrenaline.

Our observations with guanethidine, and the lack of effect of acetylcholine on the chick expansor secundariorum muscle confirm the reports of Buckley & Wheater (1968) and Buckley & Lwin (1970) that the chick expansor secundariorum muscle is a sympathetically innervated smooth muscle preparation which lacks muscarinic receptors. The poor activity of guanethidine in inhibiting noradrenergic transmission in this wing muscle at the usual incubation temperature of 23°C (Buckley & Wheater 1968) probably reflects poor uptake of guanethidine via the neuronal uptake system (which has been shown to be temperature sensitive in other tissues Dengler et al 1962)) as raising the incubation

temperature to 37°C resulted in guanethidine establishing substantial noradrenergic neurone-blockade. The inability of neostigmine to modify responses of the chick expansor secundariorum muscle to field stimulation or exogenous agonists may be attributed to the absence of postsynaptic muscarinic receptors and in turn supports the proposed postsynaptic site of action for neostigmine in the rat anococcygeus.

Byrne & Large (1987) have recently demonstrated that carbachol induces atropine-sensitive increases in ionic conductance in freshly prepared smooth muscle cells of the rat anococcygeus muscle and suggest that muscarinic receptors and adrenoceptors may activate similar membrane conductances, the most prominent being an increase in chloride ion conductance. An alternative mechanism might involve potassium channels. The 'M'-current, a neuronal K^+ efflux which is conceived to play an important role in limiting repetitive activity (Adams et al 1982), is inhibited by muscarinic agonists. The existence of a variety of K^+ channels in excitable and non-excitable cells is recognized (see Rudy 1988) and the presence of an M-channel in anococcygeus smooth muscle cells could account for the ability of neostigmine to prolong responses to field stimulation whilst failing to elicit a response in its own right.

We conclude that postsynaptic muscarinic receptors are involved in the augmentation, by neostigmine, of the excitatory effects produced by field stimulation or exogenous agonists in the rat anococcygeus muscle.

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