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The actions of some anticholinesterase drugs on skeletal muscle in culture

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The actions of the anticholinesterase drugs, physostigmine, neostigmine and diisopropylfluorophosphate (DFP) on chick embryonic skeletal muscle in culture were studied. None of the anticholinesterases potentiated depolarization responses to acetylcholine. In high concentrations neostigmine and physostigmine produced depolarization. The neostigmine-induced, but not the physostigmine-induced, depolarization was antagonized by tubocurarine. DFP caused an increase in the rate of repolarization during the presence of a cholinomimetic. It is concluded that the cholinesterase present in cultured muscle fibres does not have a physiological role in hydrolysing acetylcholine and that physostigmine and DFP have an action at the ionic channels that are linked to the cholinoreceptor.

Skeletal muscle fibres grown in cell culture respond to acetylcholine (Dryden, 1970) and the receptors are similar in properties to adult nicotinic receptors (Harvey & Dryden, 1974a,c). However, in one respect the pharmacological properties of cultured muscle appear to differ from those of normal innervated skeletal muscle: the response to acetylcholine is reported not to be potentiated by anticholinesterases (Kano & Shimada, 1971; Fischbach, 1972; Harris, Marshall & Wilson, 1973; Harvey & Dryden, 1974a).

It has been demonstrated both histochemically and biochemically that cholinesterase is present in cultured muscle (Engel, 1961; Goodwin & Sizer, 1965; Oh & Johnson, 1972; Fluck & Strohman, 1973; Wilson, Nieberg & others, 1973; Harvey & Dryden, 1974b) although its physiological significance in unknown. Before excluding a role in hydrolysing acetylcholine, it was decided to investigate further the actions of the anticholinesterase drugs, physostigmine, neostigmine and diisopropylfluorophosphate (DFP) on aneural cultures of skeletal muscle.

METHODS

Tissue culture

Monolayer cultures of skeletal muscle were obtained from the leg muscle of 10–11 day chick embryos. The culture method was identical to that of Harvey & Dryden (1974a) except that some cultures were set at the higher density of 1×10^6 cells ml^{-1}.

Electrophysiological recording and drug application

Intracellular membrane potentials were recorded using conventional glass microlectrodes filled with 3M KCl according to Harvey & Dryden (1974a). For each culture a series of control measurements were made, the medium was removed and the test solution added. Membrane potential measurements in the presence of agonist were made on random fibres throughout the test period. The values obtained in each minute were averaged and the maximum response plotted on the dose-

response curves. Each culture was used once only. All experiments were conducted at room temperature (20–22°). The responses of cultured muscle to drugs are not significantly different at this temperature from responses of conventional avian muscle preparations at 32°. Acetylcholinesterase in cultured muscle remains functional at room temperature (Harvey & Dryden, 1974b).

Cultures were incubated with the required anticholinesterase drug for 10 min before addition of agonist in the same concentration of anticholinesterase. Some cultures were exposed to $5{\cdot}4 \times 10^{-6}$M (1 μg ml^{-1}) DFP for 30 min and then maintained in Minimum Essential Medium (MEM) (Eagle, 1959) for 12 h before the responses to acetylcholine were measured.

Cultures with mean control resting potentials in the range 16–37 mV (inside negative) were used. For comparison of results, depolarization responses were expressed as per cent of control resting potential levels. The percentage depolarization of the membrane of a cultured muscle fibre to a given concentration of agonist is independent of the resting potential (Harvey & Dryden, 1974a).

Drugs and materials used

The drugs were acetylcholine chloride, (+)-tubocurarine chloride, physostigmine sulphate, neostigmine methylsulphate (all Sigma), carbachol (carbamylcholine chloride, Aldrich) and diisopropylfluorophosphate (Boots). All compounds were dissolved in MEM before adding to cultures.

RESULTS

Effect of anticholinesterase drugs on responses to acetylcholine

The effect of 10^{-7} and 10^{-8}M physostigmine and neostigmine on responses to acetylcholine is shown in Fig. 1. Physostigmine had no potentiating action at these concentrations. Neostigmine was similar except that there was a reduction in the acetylcholine response with 10^{-7}M neostigmine. At these concentrations, physostigmine and neostigmine themselves had little effect on membrane potential (Fig. 3).

In an attempt to avoid any possible desensitizing action of the anticholinesterase drugs on the acetylcholine receptors, some cultures were exposed to the irreversible

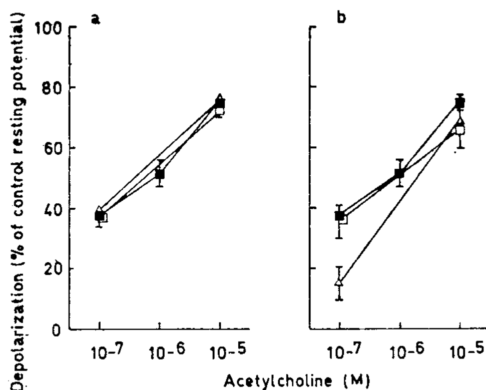

Fig. 1. Dose-response curves to acetylcholine in the presence of physostigmine (a) and of neostigmine (b). Dose-response curves in the absence of (■) and in the presence of 10^{-8}M (□) and of 10^{-7}M (△) of the respective anticholinesterase. Each point represents the mean (± s.e.) of experiments on at least 2 cultures and includes measurements of between 50–250 individual fibres.

anticholinesterase, DFP for 30 min. After 12 h the dose-response curve for acetylcholine was determined (Fig. 2a). The responses after DFP were similar to responses obtained in cultures kept for 12 h in MEM but without DFP treatment. Some cultures were stained for cholinesterase activity (Koelle & Friedenwald, 1949) after the acetylcholine response had been measured. Although there was some cholinesterase staining in the DFP-treated cultures it was very much less than in control cultures.

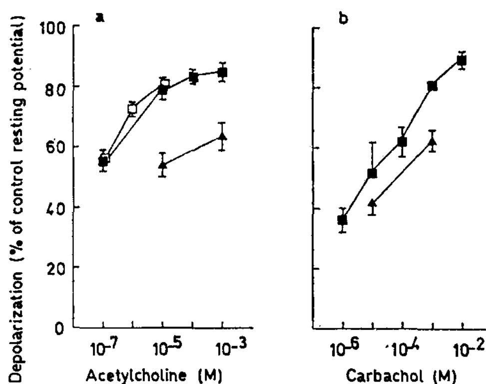

FIG. 2. Effect of DFP ($5{\cdot}4 \times 10^{-6}$M) on acetylcholine and carbachol responses. a, Dose-response curves to acetylcholine without (■) and with (□) treatment with DFP. Dose-response curve to acetylcholine *in the presence of* DFP after 10 min incubation with DFP (▲). b, Dose-response curves to carbachol in the absence of (■) and in the presence of (▲) DFP after 10 min incubation with DFP.

Depolarizing actions of the anticholinesterase drugs

The direct effects of physostigmine and neostigmine on the membrane potential were examined (Fig. 3). Both drugs caused depolarization, although they were less potent than acetylcholine or carbachol (the dose-response curve for carbachol is shown for comparison in Fig. 3a). Neostigmine was more potent than physostigmine, the concentration of neostigmine producing a depolarization 50% of

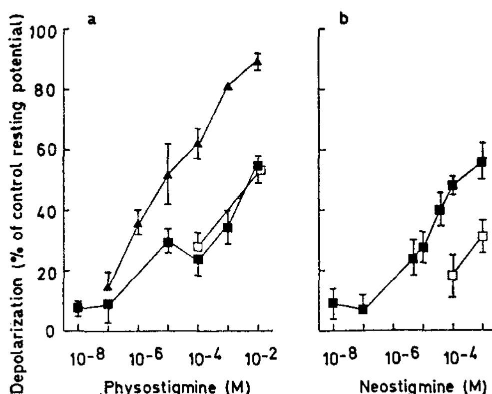

FIG. 3. Dose-response curves to physostigmine, neostigmine and carbachol. a, Physostigmine (■), carbachol (▲), and physostigmine in the presence of 10^{-4}M tubocurarine after 10 min incubation with tubocurarine (□). b, neostigmine (■), and neostigmine in the presence of 10^{-4}M tubocurarine after 10 min incubation with tubocurarine (□).

the maximal response to carbachol was about 6×10^{-5}M while the equivalent concentration of physostigmine was about 3×10^{-3}M. The depolarization by neostigmine was reduced by the presence of 10^{-4}M tubocurarine. However, tubocurarine did not affect the depolarization induced by physostigmine (Fig. 3).

The agonist actions of DFP were not investigated. DFP at $5{\cdot}4 \times 10^{-6}$M did not result in any membrane depolarization.

Effects of DFP on cholinomimetic responses

During the experiments with cultures that had been treated with DFP and allowed to recover for 12 h, it was observed that the rate of repolarization in the presence of acetylcholine was greater in the DFP cultures than in controls. Three min after adding 10^{-5}M acetylcholine to DFP cultures the membrane potential had returned to $57{\cdot}9 \pm 5{\cdot}4\%$ (mean ± s.e.) of the pre-drug level, whereas the membrane potential of controls had only returned to $39{\cdot}6 \pm 2{\cdot}8\%$ (the difference is significant, Student's *t*-test, $P<0{\cdot}001$). To investigate the possible action of DFP on the depolarization response to an agonist, cultures were incubated with $5{\cdot}4 \times 10^{-6}$M DFP for 10 min and then responses to acetylcholine and carbachol were measured in the continued presence of DFP. The effect on the depolarization produced by 10^{-3}M acetylcholine and carbachol is shown in Fig. 4. The rate of recovery, which can be taken as an indication of desensitization, was much faster with DFP present than in controls. Both acetylcholine and the cholinesterase-stable agonist, carbachol, were affected, indicating that this effect of DFP is unlikely to be related to cholinesterase inhibition.

In addition to its action on rate of repolarization DFP reduced the depolarization responses to acetylcholine and carbachol (Fig. 2a and b).

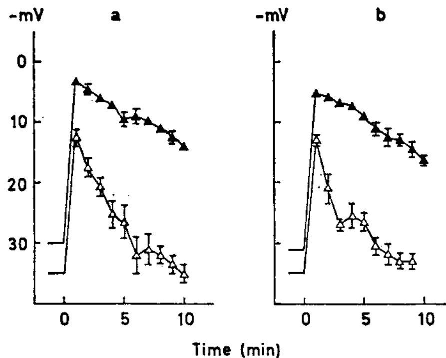

FIG. 4. Depolarization produced by acetylcholine and carbachol in the presence and absence of DFP. Ordinate gives the value of membrane potential (in negative mV); agonist was added at time 0 min. a. Response to 10^{-3}M acetylcholine in the absence of (▲), and in the presence of (△) $5{\cdot}4 \times 10^{-6}$M DFP after 10 min incubation with DFP. b. Response to 10^{-3}M carbachol in the absence of (▲), and in the presence of (△) $5{\cdot}4 \times 10^{-6}$M DFP after 10 min incubation with DFP.

DISCUSSION

It is generally accepted that most of the actions of anticholinesterase drugs are explained by their ability to prolong the effects of acetylcholine (for review, see Bowman & Webb, 1972). It has been shown in chick isolated biventer cervicis muscle that physostigmine possesses direct stimulant actions in high concentrations which are additional to the effects of cholinesterase inhibition (Baldwin & Lesser, 1971). In this study, the concentrations of physostigmine and neostigmine used

were in the range suggested by Baldwin & Lesser (1971) to cause only anticholinesterase effects. However, with these concentrations of anticholinesterases no potentiation of acetylcholine responses was found in chick skeletal muscle in culture. It has been suggested that neostigmine may desensitize cholinoreceptors and thus mask any potentiation produced by the anticholinesterase action (Harvey & Dryden, 1974a). In an attempt to avoid this possibility, cultures were treated with the irreversible anticholinesterase, DFP and the acetylcholine response measured after a period sufficient for recovery from any small amount of desensitization. However, inhibition of cholinesterase did not cause potentiation of responses to acetylcholine. In chronically denervated cat tenuissimus muscle, edrophonium caused a slight membrane depolarization and antagonized, rather than potentiated, responses to acetylcholine (Axelsson & Thesleff, 1959). Our results obtained with neostigmine, physostigmine and DFP indicate that cultured muscle is similar to denervated muscle in that there is no potentiation of acetylcholine responses, and that the cholinesterase present in cultured muscle does not have the normal physiological role of hydrolysing acetylcholine.

It has been suggested that anticholinesterase drugs exert their primary action on motor nerve terminals and that twitch potentiation is a consequence of this action (for review, see Riker & Okamoto, 1969). The absence of potentiation in aneural cultures of skeletal muscle after anticholinesterases could be taken as evidence for a prejunctional site of action.

Neostigmine is known to have agonist actions on skeletal muscle (Riker & Wescoe, 1946, Rang & Ritter, 1971) and in earlier reports it was shown that both neostigmine and physostigmine could depolarize cultured skeletal muscle fibres (Harvey & Dryden, 1974a,c). This has been examined in more detail. Neostigmine was more potent than physostigmine, the concentration of neostigmine required to produce half the maximum effect of carbachol being similar to that required in chick isolated biventer cervicis muscle ($2{\cdot}4 \times 10^{-5}$M) (Rang & Ritter, 1971). The depolarization induced by neostigmine, but not that by physostigmine, was antagonized by tubocurarine. The lack of effect of tubocurarine on physostigmine suggests that physostigmine may act at a site other than the acetylcholine receptor to produce depolarization. Similar actions of high concentrations of atropine and hyoscine have been observed (Harvey & Dryden, unpublished observations), and it is suggested that these drugs have a direct action on the ion channels linked to the cholinoreceptor, i.e. the "ion conductance modulators" (Albuquerque, Barnard & others, 1973; Kuba, Albuquerque & Barnard, 1973).

The action of DFP on the rate of repolarization in the presence of a cholinomimetic is equivalent to an increase in rate of desensitization (Harvey & Dryden, 1974a). DFP in higher concentrations increases the rate of decay of the end-plate current of frog sciatic nerve-sartorius muscle preparations (Kuba & others, 1973). These authors concluded that DFP affected the receptor-ionic conductance modulator complex, and our results would suggest that such a mechanism operates in cultured skeletal muscle.

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