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ACTION OF COLCHICINE AND CYTOCHALASIN B ON THE ACETYLCHOLINE RECEPTOR

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1 The effects of colchicine and cytochalasin B have been investigated on the extrajunctional acetylcholine (ACh) receptors of rat muscle.

2 Colchicine and cytochalasin B each decreased the amplitude and rise time of the iontophoretic ACh potential. When the dose of ACh was increased in the presence of these agents, ACh potentials were produced with a biphasic waveform consisting of a fast initial phase and a second slow phase.

3 It is proposed that colchicine and cytochalasin B are acting through a mechanism other than the breakdown of microtubules and microfilaments.

Introduction

Colchicine is an alkaloid drug which breaks down microtubules by binding to the protein tubulin (Wilson, Bamburg, Mizel, Griseham & Creswell, 1974), whereas cytochalasin B is a fungal metabolite which disrupts cell microfilaments by an unknown mechanism (Wessels, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn & Yamada, 1971). Both drugs have been used extensively in the investigation of the role of microtubules and microfilaments in cellular processes such as the mobility of cell surface receptors. For example, the application of cytochalasin B and/or colchicine to many types of cells has been found to enhance the mobility and alter the distribution of surface receptors such as immunoglobulin, concanavalin A and histamine receptors, the normal random arrangement of the receptors often being altered into patches and subsequently into caps at one end of the cell (Taylor, Duffus, Ruff & de Petris, 1971; Nicholson, 1976). These experiments have led several investigators to conclude that surface receptors span the entire plasma membrane and are connected intracellularly to microfilaments and/or microtubules (Nicholson, 1976; Edelman, 1977). The present experiments were designed to determine whether the possible intracellular attachment of microtubules and microfilaments to the acetylcholine (ACh) receptor influences the neurophysiological properties of the receptors. Although colchicine and cytochalasin B have been found to alter the properties of the receptor, we have concluded that the action of these drugs is probably not caused by the disruption of microfilaments and microtubules.

Methods

Experiments were carried out on the soleus muscles of female rats (100 to 200 g body wt.). Each muscle was denervated by removal of 0.5 to 1 cm of the sciatic nerve in the mid-thigh region. Five to 10 days after denervation, the muscle was removed and placed in a bath perfused with saline of the following composition (mM): Na^+ 135, K^+ 5, Ca^{2+} 3, Cl^- 143 and HEPES 2, pH 7.2. The saline was oxygenated with 95% O_2 and 5% CO_2. Experiments were performed at room temperature (18 to 23°C). Cytochalasin B was dissolved in 1% dimethyl sulphoxide.

Cytochalasin B, colchicine and decanol were obtained from Sigma Chemical Company.

Intracellular recordings were made with 5 to 15 MΩ microelectrodes filled with 2 M potassium citrate, and iontophoresis was performed with high resistance (100 to 200 MΩ) electrodes filled with 1 M ACh chloride. A bias current of a few nA was applied to the iontophoretic electrode to prevent diffusion of ACh from the tip of the electrode. The iontophoretic current was monitored with an operational amplifier

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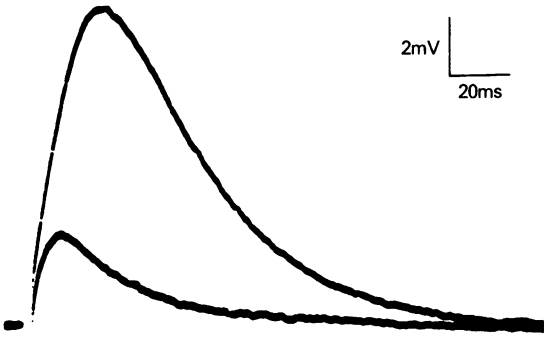

Figure 1 Effect of colchicine 2×10^{-4} M on the acetylcholine (ACh) potential evoked from extrajunctional ACh receptors of denervated rat soleus muscle. The largest ACh potential was obtained in saline, and the smallest potential in colchicine 2×10^{-4} M. Note the large reduction in the time to peak of the ACh potential.

placed between the bath and ground. The sensitivity to ACh was expressed in mV depolarization per nC of charge (Miledi, 1960).

Results

In denervated mammalian muscle, acetylcholine receptors are present over the entire surface (Axelsson & Thesleff, 1959). All the present experiments were carried out on the soleus muscle of the rat 5 to 10 days after denervation, at which time the extrajunctional ACh sensitivity to iontophoretically applied ACh was 300 to 500 mV/nC.

Perfusion of colchicine or cytochalasin B caused a decrease in the amplitude and time to peak of the iontophoretic ACh potential. Increasing the concentration of the drugs caused greater reductions in the amplitude and rise time, although the effect of these drugs showed great variability. Colchicine 1×10^{-3} M or cytochalasin B 2×10^{-4} M caused a reduction in the amplitude of the potential of over 90% and a reduction in the time to peak of 50 to 75%. Colchicine 2×10^{-4} M or cytochalasin B 8×10^{-5} M reduced the ACh potential amplitude by 25 to 75% and the time to peak by 25 to 60%. An example of the reduction in amplitude and time to peak of the ACh potential caused by colchicine 2×10^{-4} M is shown in Figure 1. These agents often took 15 to 20 min to reach a maximum effect at the concentrations described above. Prolonged exposure (2 to 5 h) of the muscles to low concentrations of these agents, such as colchicine 1×10^{-5} M or cytochalasin B 2×10^{-5} M did not produce any change in the ACh potential.

In the presence of colchicine, increasing the dose of ACh caused a drastic change of the waveform of the ACh potential. As the dose of ACh was increased, the amplitude of the ACh potential increased with only a small increase in the time to peak. However, the potential changes from being initially monophasic became increasingly biphasic with a fast initial phase

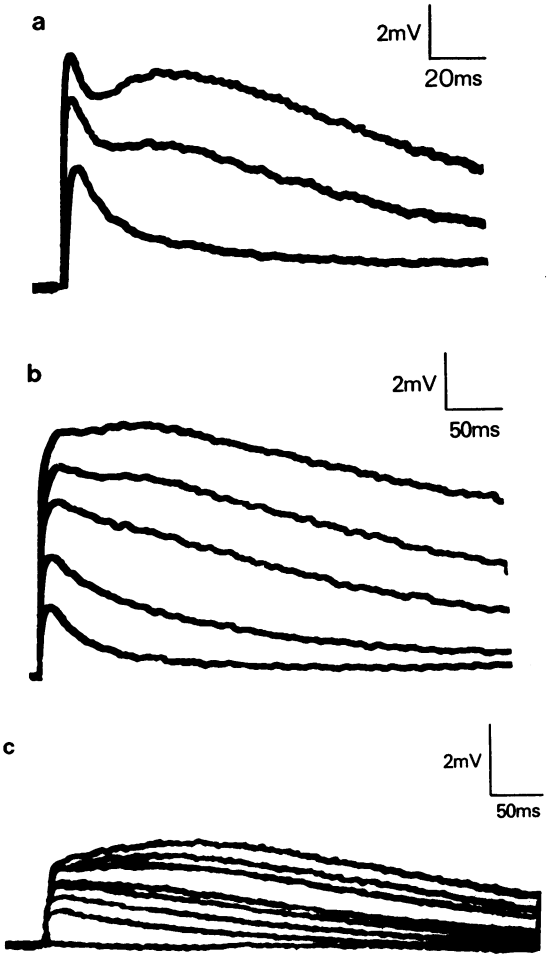

Figure 2 Effects of increasing the dose of acetylcholine (ACh) ejected from the iontophoretic electrode on the waveform of the ACh potential in colchicine 2×10^{-4} M (a and b) and in colchicine 1×10^{-3} M (c). In colchicine 2×10^{-4} M, the smallest potentials shown have a waveform similar to that in saline, whereas as the potentials increase in amplitude a second slower phase becomes increasingly pronounced. The first and second phases are sometimes distinctly separated, as in (a) but more often traces such as in (b) are obtained. In (c) colchicine 1×10^{-3} M, increasing the dose of ACh causes much less increase in the amplitude of the ACh potentials but does cause a very large prolongation of the decay phase.

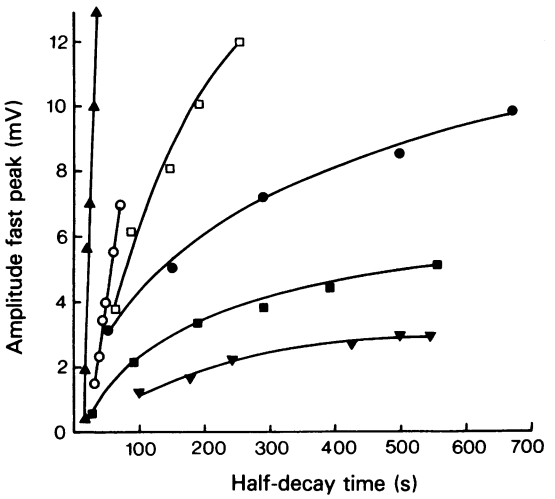

Figure 3 The increase in the half-decay times of the increasing amplitude potentials in saline (▲); (+)-tubocurarine 1×10^{-6} M (○); colchicine 1×10^{-4} M (□), 2×10^{-4} M (●), 5×10^{-4} M (■) and 1×10^{-3} M (▼).

and a prolonged later phase. Large doses of ACh often caused the amplitude of the second phase to become larger than the amplitude of the first phase (Figures 2a-c and 3).

Figure 2a and b shows the effect of increasing the dose of ACh in colchicine 2×10^{-4} M. The smallest potentials in Figure 2a and b have a waveform which is similar to that in normal saline, but as the dose of ACh is increased, the second slow phase appears and becomes more prominent. The slow peak amplitude was sometimes very pronounced and separated from the initial rapid peak by a marked fall in the amplitude of the potential (Figure 2a), but more often the peaks of the first and second phases were not separated by such a distinct dip (Figure 2b). The two peaks were more separated when the ACh potential had a faster rise time. In Figure 2b, the half decay time increased from 50 ms for the smallest (3 mV) potential to 675 ms for the largest (10 mV) potential, although the rise time only increased from 12 ms (3 mV potential) to 20 ms (10 mV potential). In higher concentrations of colchicine, increasing the amount of ACh ejected from the electrode caused a much smaller increase in the amplitude of the fast and slow phases, the main effect being to prolong the ACh potential. For example, in Figure 2c in which the preparation was perfused with colchicine 1×10^{-3} M, increasing doses of ACh increased the initial peak of the ACh potential to a maximum amplitude of 3 mV, and larger ACh doses then mainly prolonged the decay phase of the potential.

A comparison of the increases in half-decay times in normal saline and in different concentrations of

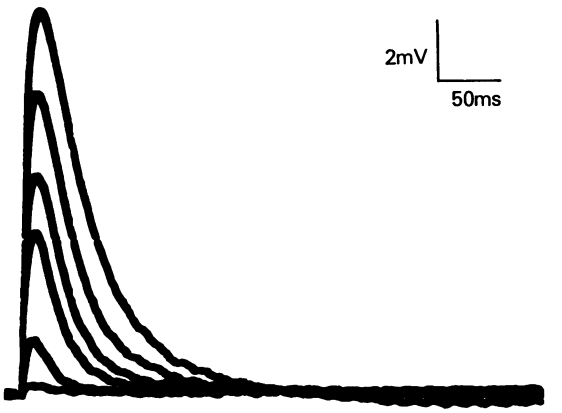

Figure 4 Effect of increasing the dose of acetylcholine (ACh) ejected from the iontophoretic electrode in saline. Note that only small increases occurred in the time to peak and the decay phase of the potential.

colchicine with increasing dose of ACh is shown in Figure 3. It can be seen that the increase in half-decay time for a given increase in the amplitude of the potential becomes larger as the concentration of colchicine is raised. The half-decay time increased by 1.7, 12, 56, 100 and 170 ms per mV increase in ACh amplitude in saline, 1×10^{-4} M, 2×10^{-4} M, 5×10^{-4} M and 1×10^{-3} M colchicine respectively.

Cytochalasin B caused similar biphasic responses to colchicine when the dose of ACh was increased, although the biphasic waveforms were less pronounced than in colchicine.

It was considered possible that the change in shape of the ACh potential in the presence of colchicine and cytochalasin B was an artefact caused by diffusion of ACh to different areas of receptors, such as receptors close to and distant from the tip of the iontophoretic electrode. Such an effect would be particularly pronounced with extrajunctional receptors which are present at a much lower density than junctional receptors. The possibility of such an artefact was excluded by the following control experiments.

Firstly, the dose of ACh was increased in saline (Figure 4). This caused a small increase in the time to peak and half-decay time of the ACh potential, but biphasic potentials were never observed even when the amplitude of the ACh potential was 20 to 30 mV (the dose of ACh evoking such large ACh potentials was comparable to that causing biphasic potentials in the presence of colchicine).

Secondly, the action of colchicine and cytochalasin B was compared to the competitive antagonists (+)-tubocurarine and α-bungarotoxin. Curare 1×10^{-6} M reduced the ACh potential by more than 90% and increased the rise time of the potential by 10 to 20% (Figure 5a). Thus small (1 to 2 mV) ACh

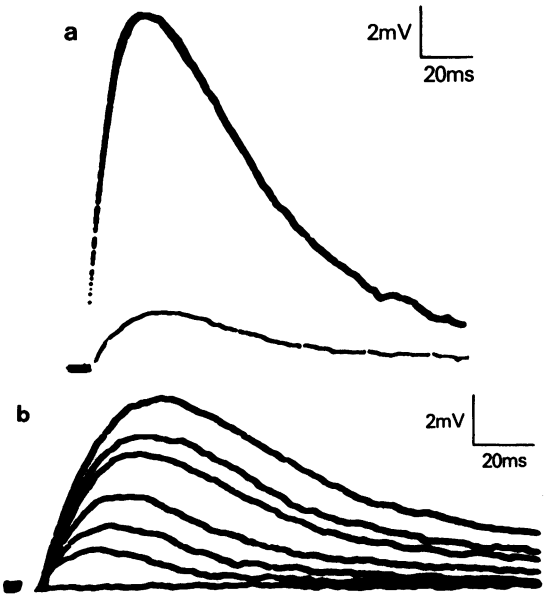

Figure 5 (a) Reduction of the amplitude of the acetylcholine (ACh) potential by curare 1 ± 10^{-6} M. Large potential is in normal saline, and small potential is in 1×10^{-6} M curare. A small increase in the rise time occurred in curare. (b) Effect of increasing doses of ACh in presence of curare (1×10^{-6} M). A larger increase in rise time and much smaller increase in half-decay time occurred than with the other agents investigated.

potentials in curare 1×10^{-6} M had rise times at least twice as great as in concentrations of colchicine and cytochalasin B which caused a similar reduction in ACh sensitivity. When the dose of ACh was increased in curare 1×10^{-6} M, the rise time increased by a larger amount and the decay time by a much smaller amount than that occurring with the other drugs investigated (Figures 3 and 5b). No biphasic responses occurred in curare. ACh potentials in the presence of α-bungarotoxin (0.5 μg/ml) were similar to those in (+)-tubocurarine i.e. ACh potentials evoked by large doses of ACh had very slow rise times (up to 100 ms) and very slow half-decay times, but were never biphasic.

Thirdly, the action of colchicine and cytochalasin B was compared to that of the barbiturate, sodium thiopentone, and the alcohol, decanol. The action of barbiturates on the ACh receptor has been previously investigated (Adams, 1976). Sodium thiopentone and decanol were found to have very similar effects to colchicine and cytochalasin B, reducing the amplitude and rise time of the ACh potential evoked by a constant iontophoretic current. Sodium thiopentone 1×10^{-4} M and a $1:10^4$ mixture of decanol: saline caused a reduction in the amplitude of the potential of over 40% and a reduction in the time to peak of about 50%. Increasing the dose of ACh in the presence of sodium thiopentone or decanol caused biphasic ACh potentials with the second phase being very pronounced (Figure 6).

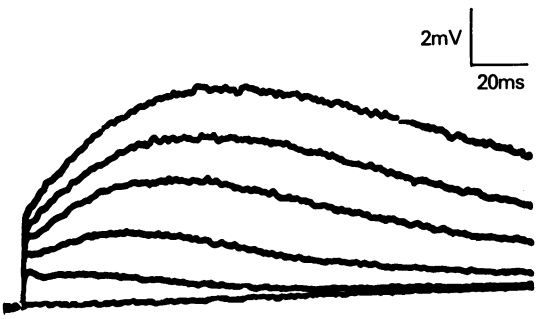

Figure 6 Effect of increasing the dose of acetylcholine (ACh) in decanol ($1:10^4$ mixture of decanol:saline). Note the very large amplitude of the second phase in decanol. Almost identical traces were obtained in sodium thiopentone 8×10^{-5} M.

Discussion

The experiments described in the present study were designed to investigate the role of microtubules and microfilaments in the functioning of the ACh receptor by the use of colchicine and cytochalasin B which have been used extensively to disrupt microtubules and microfilaments (Nicholson, 1974). Colchicine and cytochalasin B were found to have a large effect on the neurophysiological properties of the ACh receptor, causing a decrease in the sensitivity to ACh and also an alteration of the waveform of the ACh potential into fast and slow phases. These effects are very different from those of the competitive antagonist curare, which reduced the amplitude of the ACh potential but did not produce any biphasic responses. Curare has a similar action on the ACh receptors at the frog neuromuscular junction (Steinbach, 1968).

It is possible that microtubules and microfilaments are associated intracellularly with the ACh receptor, as postulated for many other types of cell surface receptors (Nicholson, 1976; Edelman, 1977), and that breakdown of either of these elements by colchicine and cytochalasin B results in the altered waveform of the ACh potential. However, there are several reasons for believing that this is an unlikely explanation. The concentrations of colchicine and cytochalasin B which were effective in producing a biphasic ACh potential are 10 to 100 times greater than those needed to disrupt microtubules and microfilaments. Moreover, the action of colchicine and cytochalasin B was dose-dependent at least up to concentrations of 10^{-3} M in the present study, whereas the cytoskeletal disruption by these drugs has a maximum effect at 10^{-6} M to 10^{-7} M (Samson, 1976; Riordon & Alon, 1977). These recent biochemical studies have shown

that colchicine and cytochalasin B bind non-specifically to cell membranes at concentrations above 10^{-5} M with the binding being concentration-dependent. Further evidence against a microtubule disrupting effect of colchicine being responsible for the altered ACh responses is that it has been shown that chick muscle cells cultured in low concentrations of colchicine (10^{-8} M) are devoid of microtubules although they still have normal ACh sensitivity (Fukuda, Henkart, Fischbach & Smith, 1976). The finding that the barbiturate, sodium thiopentone and the long chain alcohol, decanol, have a very similar action to colchicine and cytochalasin B also strengthens the hypothesis that the properties of the ACh receptor are being altered by a mechanism other than the breakdown of microtubules and microfilaments.

Previous studies on the frog neuromuscular junction have shown that barbiturates reduce the time to peak of the endplate currents and cause ACh potentials produced by large doses of ACh to become biphasic (Adams, 1976). Local anaesthetics have also been shown to produce biphasic ACh potentials (Katz & Miledi, 1975). However, local anaesthetics must have a somewhat different action from barbiturates as unlike barbiturates, they produce biphasic endplate currents (Kordas, 1970). It must be emphasized that the decay time course of the endplate current is determined mainly by the conductance properites of the endplate channels, with the ACh concentration being near zero. However, the decay time course of the ACh potentials is determined by the conductance properties of the channel and by the variation of ACh concentration with time. Thus analysis of the ACh potential time course is complicated. In the present paper, detailed control experiments in saline, curare and α-bungarotoxin were carried out to ensure that the biphasic potentials observed in colchicine, cytochalasin B, sodium thiopentone and decanol were not caused by the diffusion of ACh to different areas of receptors.

The similarity in the action of colchicine, cytochalasin B, barbiturates, long chain alcohols and local anaesthetics suggests that all these agents may have a common mechanism of action. Recent studies on single channel noise have shown very conclusively that local anaesthetics cause the open channel to become blocked in a transient manner (Steinbach, 1977). It has been postulated from these and other noise studies that the local anaesthetics interact with the ACh receptor after the channel has been opened by ACh, with the action being described by a sequential kinetic binding scheme (Steinbach, 1977; Ruff, 1977).

$$A + R \underset{R_2}{\overset{R_1}{\rightleftharpoons}} AR^* + Q \underset{R_4}{\overset{R_3}{\rightleftharpoons}} AR'Q$$

R is the ACh receptor, R* is the receptor with an open channel, R′ is the open channel with greatly reduced conductance, A is the agonist, and Q is the local anaesthetic. It has also been proposed that anaesthetic-like agents interact with membrane lipids or lipid-receptor interaction, thus altering the membrane dielectric constant and membrane fluidity (Gage & Hamill, 1976), the membrane lateral phase separation (Trudell, 1977), or the state of the lipid annulus around the receptor protein (Lee, 1976). Thirdly, it has been proposed that anaesthetics disrupt the cytoskeletal elements which, it has been suggested, are associated with membrane proteins (Poste, Papahadjopoulos, Jacobson & Vail, 1975). Local anaesthetics have been found to have a similar action to colchicine and cytochalasin B in altering the distribution of cell surface receptors such as concanavalin A (Poste *et al.*, 1975). Moreover, electron microscope studies have shown that local anaesthetics do break down microtubules and microfilaments (Poste *et al.*, 1975). However, we believe that it is unlikely that the biphasic ACh potentials are produced by breakdown of cytoskeletal elements for the reasons discussed earlier.

Although the neurophysiological data are consistent with anaesthetic type agents acting by causing a block of the open channel, such neurophysiological studies cannot determine the binding site of the anaesthetic. For example, anaesthetic bound to membrane lipids or to a site on the actual ACh receptor could cause the channel to become blocked in a transient manner only after it had been opened by ACh. Thus, although binding of the anaesthetic occurs before activation of the receptor by ACh, as in a parallel kinetic scheme, the binding of the anaesthetic only changes the responses after the channel has been opened by ACh, and therefore such a theory would be equivalent to the sequential kinetic scheme. The results of the present investigations favour the theory that substances producing the biphasic responses are binding in a non-specific manner to hydrophobic sites on the ACh receptor or membrane lipids. It has been shown that cytochalasin B and colchicine bind non-specifically to cell membranes, probably by partitioning into the lipid phase, cytochalasin B having a high partition coefficient of 60 (Hauschka, 1973; Riordan & Alon, 1977). The binding could specifically affect lipid-protein interactions which have been shown to be very important in the functioning of membrane proteins such as the calcium transporting protein (Warren, Houslay, Metcalfe & Birdsall, 1975) and rhodopsin (Hong & Hubel, 1972).

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