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Modification of drug responses by hydrolytic Enzymes

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α-Chymotrypsin and phospholipase enzymes have been used to alter the cell membranes of the smooth muscle of the guinea-pig taenia coli. The effects of these treatments on the electrical and mechanical responses of the muscle to acetylcholine have been studied. Prolonged treatment with both enzymes causes depolarization with a consequent loss of acetylcholine sensitivity. After controlled trypsinization a membrane response to acetylcholine was obtained in the absence of a mechanical response. The sensitivity of the taenia coli to acetylcholine was depressed by controlled phospholipolysis without affecting the membrane potential or contractile apparatus. The implications of these findings are discussed.

IT is generally supposed that drug receptors in smooth muscle are specialized protein molecules located on the cell membrane. These are considered to undergo a conformational or other change on reaction with the drug resulting in permeability or other changes which in their turn lead to the mechanical response.

Recently suggestions have been made that a phospholipid component of the cell membrane is implicated in chemoceptive action. Dikstein & Sulman (1965a) found that when rabbit aortic strips were exposed to labelled dibenamine the drug was bound to a cephalin fraction. Further, the binding of dibenamine was prevented by adrenaline. The same authors (Dikstein & Sulman, 1965b) also found that the responsiveness of the frog rectus and rabbit uterus preparations to drugs was reduced by treating with 25% acetone for 2 min and that reactivity in these preparations was restored by treating the preparations with phosphatidylethanolamine or phosphatidylserine. Woolley & Gommi (1964) found that the combined action of neuraminidase and EDTA on rat stomach strips selectively inhibited the action of 5-hydroxytryptamine (5-HT). Sensitivity to this agent was quickly restored by adding a crude extract of stomach lipids and the authors suggested that the 5-HT receptor is a neuraminidase-sensitive ganglioside.

In this paper hydrolytic enzymes have been used to alter the protein or lipid components of the cell membrane (Tobias, 1958) and the effect of this treatment on the responses to drugs, particularly acetylcholine, has been investigated.

Experimental

MATERIALS AND METHODS

The preparation used was the isolated taenia coli of the guinea-pig. The physiological saline solution employed throughout was a Krebs solution of the following composition: (mM) NaCl, 118; KCl, 4·7; $CaCl_2$ 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 25; NaH_2PO_4, 1·1; and glucose, 5·6. Preparations were mounted either in an isolated organ bath maintained at 37° and gassed with a mixture of oxygen 95% and carbon dioxide 5%, or in a sucrose gap electrode at 37°. The latter was of conventional design and provision was made for recording the isometric tension of the preparation simultaneously with the electrical membrane activity. The catecholamine

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content of guinea-pig hearts was estimated fluorimetrically by a method based on that devised by Euler & Lishajko (1961).

DRUGS AND ENZYMES

Drugs were given in two ways to preparations mounted in the sucrose gap electrode. Either the Krebs solution flowing through the apparatus was changed to Krebs solution containing dissolved drug or the drug was injected, in a small volume of Krebs solution, into the fluid perfusing the tissue. The following two enzymes were used; α-chymotrypsin (EC 3.4.4.5, Seravac Laboratories Ltd., salt free with an activity of 11,000 ATEE u/mg) and phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3, Koch-Light Laboratories Ltd., from *Clostridium welchii*).

Results and discussion

When guinea-pig taenia coli, mounted in an organ bath, were exposed to phospholipase C or to α-chymotrypsin the responses of the muscle strips to acetylcholine were eventually abolished. In the case of the phospholipase enzyme, a 2 hr exposure to a concentration of 20 μg/ml was sufficient to abolish the response to acetylcholine. The reduction in the sizes of the responses during exposure to the enzyme was gradual and showed no unusual features. In some experiments there was a small increase in tone of the muscle strip following addition of the phospholipase. With α-chymotrypsin (0·5 or 1·0 mg/ml) the results were more complicated. The enzyme itself caused a large increase in tone which then declined, during 1–2 hr, to a level lower than that existing before exposure to the enzyme. During the first hour of exposure the response to acetylcholine was still present, although reduced during the period of raised tone. Then, suddenly, addition of acetylcholine caused the muscle to relax. These inhibitory responses could be obtained three or four times during 20 min and showed tachyphylaxis. Eventually, as with phospholipase C, after about 2 hr no response was obtained to acetylcholine. Inhibitory responses to acetylcholine after exposure to chymotrypsin were not obtained in preparations taken from guinea-pigs given 5 mg/kg reserpine 18 hr previously. The hearts from such animals were found to be 95% depleted of catecholamines and it is concluded that the inhibitory responses of the taenia to acetylcholine were the result of catecholamine release.

The experiments described above were repeated with taenia coli preparations mounted in a sucrose gap electrode so that both tension and electrical membrane responses could be examined. In these results the membrane potential of the tissue is taken as the difference in potential between the active part of the tissue, perfused in warmed Krebs solution at 37°, and the inactive part, perfused in either isotonic K_2SO_4 or isotonic KCl. Such a measure is inaccurate due to the various junction potentials existing between Krebs solution and sucrose, sucrose and K_2SO_4 or KCl, and between the KCl agar of the recording electrodes and the solutions with which they are in contact. These junction potentials may be large

but only a fraction of them will be recorded depending on the short circuiting factor of the gap, that is, the relative impedences offered by tissue compared with the surrounding sucrose solution. In spite of these drawbacks, changes in membrane potential as a result of enzymic and drug treatment will be true as the contribution of the junction potentials to the measured potential will be constant. It is for these reasons that changes in membrane potential have been stated rather than values of the potential.

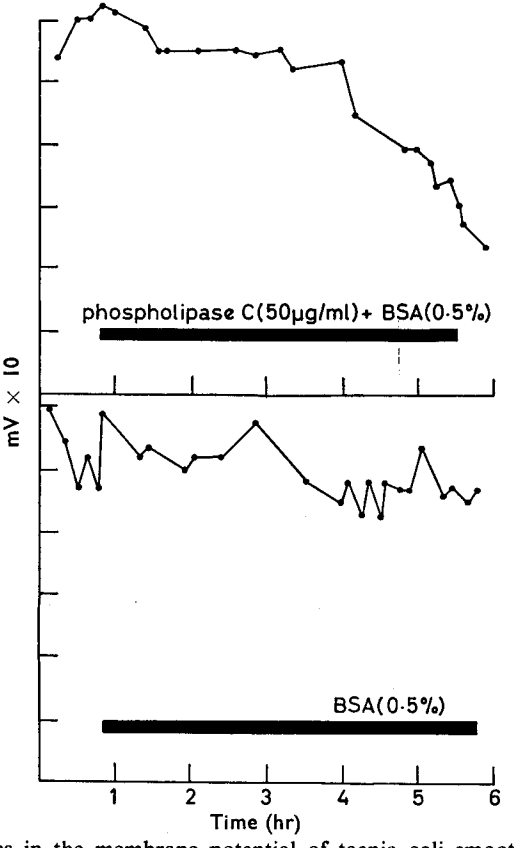

FIG. 1. Changes in the membrane potential of taenia coli smooth muscle strips exposed to phospholipase C (50 µg/ml) + 0·5% Bovine Serum Albumin (BSA) and 0·5% BSA alone, for the periods indicated by the horizontal bars.

Higher concentrations and longer exposures to phospholipase C were required to abolish the response to acetylcholine when taenia coli were placed in the sucrose gap electrode rather than in an organ bath. This is undoubtedly due to inactivation of the enzyme during its flow through the narrow bore tubes of the apparatus. It is known that the enzyme is inactivated at interfaces and particularly by bubbling (Macfarlane & Knight, 1941). Inactivation was kept to a minimum by vigorously bubbling the Krebs solution with oxygen 95% and carbon dioxide 5%

before dissolving the enzyme and by sometimes adding 0·5% bovine serum albumin (BSA) to the solution.

Fig. 1 shows the changes in membrane potential caused by exposure of a muscle strip to phospholipase C (50 μg/ml) for 6 hr. The membrane potential remained steady for the first 4 hr after which rapid depolarization took place, as would be expected if there was a sudden collapse of the membrane structure. The total fall in membrane potential was around 35 mV so that the muscle strip was 70% depolarized, assuming a resting membrane potential of 50 mV (Burnstock & Prosser, 1960). A control preparation exposed to exactly the same conditions but without phospholipase showed only a minor reduction in resting membrane potential in 6 hr (Fig. 1). Acetylcholine, which normally produces a discharge

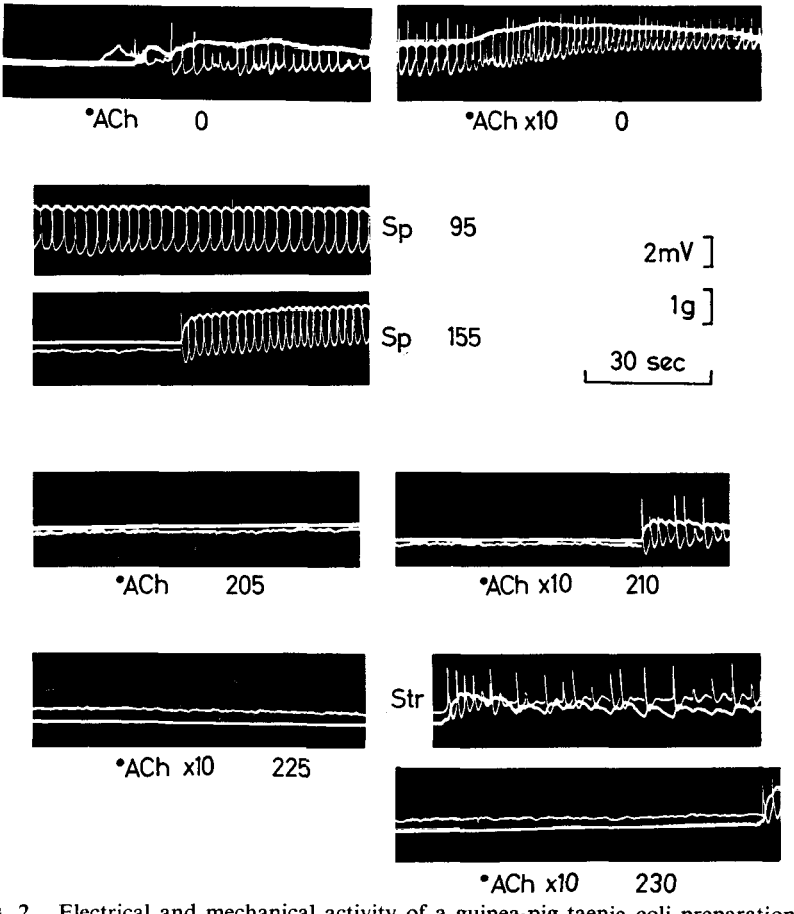

FIG. 2. Electrical and mechanical activity of a guinea-pig taenia coli preparation. The figures by each panel refer to the time, in min, during which the preparation was exposed to phospholipase C (30 μg/ml). At ACh the preparation was exposed to 0·1 μg/ml acetylcholine for 15 sec, at ACh × 10 the concentration was 1·0 μg/ml. Sp refers to spontaneous activity and Str refers to a stretch sufficient to raise the tension 0·1 g.

of action potential spikes, membrane depolarization and a sharp rise in tension was ineffective on preparations which had been depolarized by 3·5–4 hr treatment with phospholipase C. On normal preparations, potassium ions produced responses qualitatively similar to acetylcholine but in the phospholipase-treated preparation potassium ions produced only a residual depolarization unaccompanied by any spike potentials. On the other hand, caffeine (1 mg/ml) produced a contractile response in phospholipase treated muscles without any membrane response. This is in accord with the idea that caffeine liberates bound calcium from the cell membrane (Herz & Weber, 1965) and that this occurs just as well in depolarixed tissues (Axelsson & Thesleff, 1958). As might be expected the phospholipase depolarized muscles were insensitive to a stretch stimulus. Control preparations which had not been subjected to phospholipase treatment responded normally to drugs and stretch 5 hr after mounting in the apparatus.

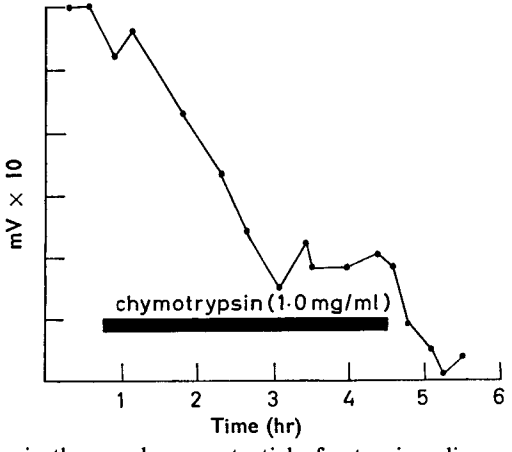

FIG. 3. Changes in the membrane potential of a taenia coli smooth muscle strip exposed to α-chymotrypsin (1 mg/ml) for the period indicated by the horizontal bar.

So far, the consequences of depolarization by phospholipase C are predictable and of little interest. However, by controlled phospholipase treatment, effects on drug responses were obtained at a time at which the membrane remained polarized. This phenomenon is illustrated in Fig. 2. Responses of a taenia strip to two concentrations of acetylcholine were determined after which phospholipase C (30 μg/ml) was added to the perfusion fluid. The preparation was spontaneously active, at first continuously and then intermittently. This is illustrated by recordings taken after 95 and 155 min exposure to phospholipase C. After some 200 min exposure to the enzyme bursts of spontaneous activity were less frequent but the membrane potential remained normal. The measured potential difference being 60 mV after 200 min exposure to the enzyme compared with values ranging from 45 to 58 mV at the beginning of the experiment. The responses to acetylcholine after 200 min of enzyme treatment are shown in Fig. 2, where it can be seen that no response was

obtained to the low concentration and a delayed response to the high drug concentration. It is impossible to tell whether this is really a delayed response or a burst of spontaneous activity commencing 45 sec after the application of the drug. After 225 min and 230 min of enzyme treatment no response was obtained even to the higher concentration of acetylcholine. At this time however the membrane was still excitable as shown by the burst of electrical and mechanical activity caused by applying a slight stretch sufficient to raise the tension in the muscle by 0·1 g.

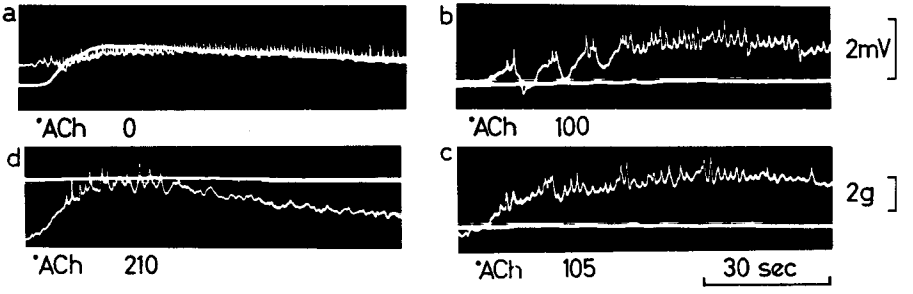

FIG. 4. Electrical and mechanical activity of a guinea-pig taenia coli preparation. At ACh in (a), (b) and (c) the preparation was exposed to acetylcholine (1 μg/ml) for 10 sec and at ACh in (d) to 10 μg/ml for 10 sec. The figures below each panel refer to the time, in min, after which treatment of the preparation with α-chymotrypsin (1 mg/ml) was commenced. The preparation was returned to normal Krebs solution after it had been perfused with the enzyme for 100 min. Note in (d) the tension on the preparation has been raised.

Turning to the effects of α-chymotrypsin on the taenia coli preparation, it was again found that prolonged exposure of the tissue to the enzyme resulted in depolarization with a consequent loss of sensitivity to drugs. Fig. 3 shows the change in membrane potential caused by exposure to α-chymotrypsin (1 mg/ml) for 4 hr. As can be seen the membrane potential fell continuously over this period and continued to do so after the enzyme was removed. The responses of the tissue became progressively more feeble as depolarization proceeded. When the membrane potential had fallen by about 40 mV the responses to acetylcholine or potassium ions consisted only of a few isolated action potentials. After a further fall in membrane potential no further responses could be elicited.

As with phospholipase, interesting and unpredictable results were obtained by controlled treatment with α-chymotrypsin. Treating taenia coli with α-chymotrypsin for periods shorter than those required to significantly reduce the membrane potential, abolished the mechanical response without much effect on the membrane changes. This type of result is illustrated in Fig. 4. In this instance the preparation was treated with α-chymotrypsin for 100 min and then returned to normal Krebs solution. Before treatment, acetylcholine produced the usual electrical and mechanical responses but at the end of, and after enzyme treatment, identical acetylcholine concentrations produced only an action potential discharge and depolarization without mechanical response. It is obvious from Fig. 4 that the nature of the electrical discharge has changed after enzyme

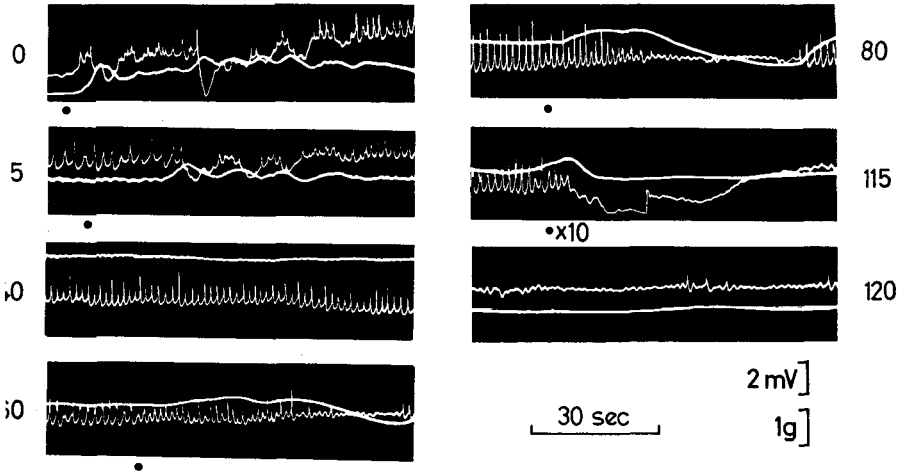

FIG. 5. Electrical and mechanical activity of a guinea-pig taenia coli preparation showing the inhibitory response to acetylcholine occurring during treatment with α-chymotrypsin (0·5 mg/ml). The duration of perfusion with α-chymotrypsin is shown (in min) at the side of each panel. At each dot the preparation was exposed for 15 sec to acetylcholine at a concentration of 1 μg/ml or 10 μg/ml (× 10). Note that this latter dose caused cessation of activity for 5 min. Following this dose the response to acetylcholine reverted to excitation.

treatment, the spikes having a slower rate of rise and fall. However this technique is not an appropriate tool for the study of spike configuration and it must suffice to point out that the character of the discharge was abnormal. It was a possibility that the enzyme treatment had so disorganized the tissue that it was no longer under an appropriate tension to show a mechanical response. Accordingly, the tension was artificially raised, but the same result was obtained (Fig. 4d). In other experiments the same uncoupling phenomenon was shown for potassium ions. From the results with acetylcholine it can be concluded that at least some muscarinic receptors were intact and functional after enzyme treatment although the normal contraction was not obtained.

As was pointed out earlier, some preparations responded to acetylcholine during α-chymotrypsin treatment with a relaxation rather than a contraction. The genesis of this effect is illustrated in Fig. 5. After 60 min tryptic digestion a biphasic response to acetylcholine was obtained, and similarly at 80 and 115 min with the inhibitory effect becoming more pronounced. The inhibition of spike activity, hyperpolarization and relaxation seen in Fig. 5 are typical of the response of the taenia coli to catecholamines and it is considered that acetylcholine is here acting by releasing catecholamines, probably from adrenergic nerves. Acetylcholine is thought to release catecholamines from adrenergic nerves (see Burn & Rand, 1965 for references) and it would appear that trypsinization of the taenia coli facilitates this. Fig. 5 also illustrates the increase in tone and electrical discharge caused by α-chymotrypsin alone. Trypsin produces a contracture in many smooth muscles and is thought to do so by the release of spasmogenic polypeptides or histamine or both (Rocha

e Silva, 1956). The stimulatory effect of α-chymotrypsin may result from a similar mechanism.

Conclusions

As was anticipated, this study has not been able to prove the chemical nature of the muscarinic receptors in smooth muscle. Two unexpected findings however emerge from the results. First, the muscarinic receptor is sufficiently resistant to tryptic digestion to remain excitable at a time when the contractile response has been abolished. Second, controlled phospholipolysis inhibits the action of acetylcholine while the membrane and contractile apparatus remain functional. Each finding is capable of many interpretations. The first may be interpreted thus: (i) the acetylcholine receptor is not protein, (ii) the acetylcholine receptor is protein but is not hydrolysed by α-chymotrypsin, (iii) α-chymotrypsin has attacked the coupling mechanism, (iv) the configurations of the action potentials are so changed that they no longer activate the coupling mechanism and (v) the contractile apparatus is inactivated. The second finding may be interpreted to mean that (i) the acetylcholine receptor is a phospholipid or (ii) phospholipids are involved in coupling receptor activation to the permeability change of the membrane.

It is known that agonist drugs, like acetylcholine, can cause enormous changes in ion permeability in smooth muscle, including the taenia coli (Burgen & Spero, 1966). It seems impossible that these almost explosive changes do not involve the lipids of the membrane if the Danielli-Davson (1935) concept of the cell membrane is accepted. Large permeability changes would seem to require a change in membrane ultrastructure with the creation of pores through which the lipid-insoluble ions can pass. This work has shown that the excitation of smooth muscle by acetylcholine is dependent on the integrity of some membrane lipids, as indicated by the decreased sensitivity shown to acetylcholine by preparations treated with phospholipase C. What is not known is whether drugs, like acetylcholine, affect a conformational change in the membrane protein which then causes a second order rearrangement of the membrane lipids or whether the drug interacts directly with the lipids. This vital question remains to be answered.

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