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Ionic interactions in acetylcholine contraction of the denervated rat diaphragm

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1. The nature of the drug-receptor interaction in the acetylcholine-induced contraction of the denervated rat diaphragm was studied both by altering the external ionic environment and by determining its drug sensitivity.

2. The response to acetylcholine was insensitive to tetrodotoxin or saxitoxin, but was abolished by procaine.

3. It was unaffected by levels of $MnCl_2$ sufficient to block the response of the innervated diaphragm to electrical stimulation, although higher levels reduced the response. The effect of Mn^{++} on the innervated diaphragm was overcome by raising the external Ca^{++} level; this was ineffective in the denervated preparation.

4. In spite of its insensitivity to tetrodotoxin the acetylcholine contraction was reduced and prolonged by low external Na^+ levels. This prolongation was not found when Li^+ substituted for Na^+.

5. Increasing the external level of Ca^{++} or Mg^{++} 3 to 5-fold reduced the acetylcholine contraction; high Ca^{++} also prolonged it. Reduction in the divalent cation level was without effect.

6. Procaine inhibition of the acetylcholine response was largely competitive, as was inhibition due to (+)-tubocurarine. This was shown by probit analysis and the dose-ratio test.

7. Thiocyanate (12 mM) augmented and prolonged the contraction; this action was modified by altering the Ca^{++} or Mg^{++} level of the solution.

8. The acetylcholine receptor resembles that of the innervated postsynaptic membrane.

The pharmacology of the acetylcholine-induced contraction of denervated muscle is of interest both for the observation of denervation phenomena and because the nicotinic receptor may be conveniently studied in such a preparation. Axelsson & Thesleff (1959) noted that denervated muscle responded to drugs in a manner qualitatively similar to the innervated endplate. Beránek & Vyskočil (1967) reported, however, that the acetylcholine (ACh) receptor of denervated muscle is less sensitive to (+)-tubocurarine (TC) blockade than is the innervated endplate, and Loomis & Konker (1967) reported that TC produces a contracture of the intact

denervated anterior tibial muscle of the rat. This finding confirmed the earlier report of McIntyre & King (1943).

It is of interest, therefore, to study the ACh receptor of the denervated rat diaphragm in an attempt to extend the observations of del Castillo & Katz (1954, 1955) on the post-synaptic ACh receptor, and to determine wherein the denervated preparation differs from it. To this end the effects of changes in the ionic environment on the ACh-induced contraction and interactions between ACh and a number of anticholinergic drugs have been studied.

Methods

Male hooded rats of the Wistar strain were anaesthetized with pentobarbital 40–50 mg/kg and the left diaphragm was denervated by evulsion of the phrenic nerve in the neck. They were maintained postoperatively for 8–21 days, to allow the nerve to degenerate. The ACh-induced contraction was constant during this period, as noted by Elmqvist & Thesleff (1960). The animals were then killed by a blow on the head, exsanguinated, and the innervated and denervated diaphragms set up as described previously (Freeman, 1968). Isometric tension developed by the muscles in response to drugs or electrical stimulation was recorded with Statham UC2 transducing cells and a Beckman type R dynograph recorder. Innervated preparations were stimulated either directly or through the nerve with 0·1 msec rectangular pulses of supramaximal voltage.

The standard nutrient solution used has been described elsewhere (Freeman, 1968). Na^+-free solutions were buffered with tris(hydroxymethyl)aminomethane hydrochloride (Tris). They were aerated with oxygen rather than the 95% oxygen and 5% carbon dioxide mixture used in the standard solution. Drugs were injected into the stream of gas bubbles which was used to aerate and to stir the organ baths. The bath temperature was monitored continuously with a thermistor. Unless otherwise stated experiments were carried out at 29° ±0·2° C.

The thickness of the muscles was determined at the end of experiments from their weight and surface area, assuming a density of 1·05. The mean thickness was 0·63 mm, and the range was from 0·58 to 0·74 mm. This agrees with our previous estimate of 0·66 mm (Freeman, 1968) and indicates that at the time of measurement denervation had not altered the thickness of the muscles. The contractile response to ACh or carbamylcholine was constant over a 5 hr experimental period, and tachyphylaxis was not evident provided each drug addition to the organ bath was separated by a 10 min wash period. Typical dose-response curves are shown in Fig. 2. The slope varied slightly from one preparation to another, consequently whenever possible each preparation was used as its own control.

TABLE 1. *Time course of acetylcholine or carbamylcholine contraction of denervated diaphragm*

	Rise time (sec)	Time to half relaxation (sec)	Maximum tension (g)
ACh $5·5\times10^{-6}$M	12·0±0·4 (45)	46±2 (45)	5·5±0·3 (45)
Carbachol $7·3\times10^{-5}$M	45±4 (18)	52±3 (18)	7·9±0·4 (18)

Figures shown are ± S.E. of the mean. The number of observations is shown in parenthesis.

Results

Time course of the contractile response

Acetylcholine or carbamylcholine (carbachol) produced a dose-dependent increase in isometric tension in the denervated diaphragm. The dose-response curves for the two drugs were parallel, but 7–9 times more carbachol was needed to produce the same tension as ACh. The response to carbachol was, however, more prolonged. As can be seen from Table 1 this resulted more from a slower rate of rise of the carbachol contraction than from a prolongation of the time to half relaxation. The tension developed in response to either drug declined as an exponential function of time.

The time course of the ACh contraction was relatively independent of the concentration of ACh used. The rise time increased slightly over the range $2{\cdot}75 \times 10^{-6}$M to $2{\cdot}75 \times 10^{-5}$M, but the time to half relaxation fell with increasing ACh concentration from 64 sec at $2{\cdot}75 \times 10^{-6}$M to 31 sec at $2{\cdot}75 \times 10^{-5}$M.

The reason for the brevity of the ACh response is obscure. Lüllmann & Reis (1967) noted that the depolarization of the denervated diaphragm due to ACh persisted after the muscle had relaxed. Consequently it cannot be ascribed to membrane "desensitization," but is more likely related to a resequestration of reticular Ca^{++}.

The duration of the contraction must also be viewed in relation to the rate of diffusion of ACh through the preparation. Krnjević & Mitchell (1960) noted that the time of half clearance of ACh from the diaphragm was 1·5 min. Their preparations appear to have been 50% thicker than ours, but it is obvious that the ACh contraction is too brief for ACh to have diffused uniformly through the muscle. One must assume that the measured contraction is a sum of contractions and relaxations which occur as ACh diffuses through the preparation. Because of this, conditions which prolong or shorten the contractile event will cause an apparent increase or decrease in the peak tension developed. This effect may partly explain the finding of Letley (1960), which was confirmed by us, that the ACh contraction develops greater tension at 22° C than at 37° C. We observed that low temperature markedly prolonged the contraction.

Effect of alteration of the external ionic environment

Castillo & Katz (1955) and Jenkinson & Nicholls (1961) noted that ACh was able to decrease the membrane resistance of the endplate and denervated muscle respectively in preparations soaked in Na^+-free solutions. Castillo & Katz (1955) described this effect of ACh "as a short-circuit placed across a rectifying membrane," and concluded that the action of ACh is independent of the process of electrical excitation. If this is so the decrease in membrane resistance in Na^+-free solution will be associated with currents due to ions other than Na^+.

Sodium substitution with sucrose

The denervated diaphragm offers a convenient preparation in which to study the effect of Na^+ depletion on the response to ACh. Sucrose, lithium or Tris hydrochloride were used to maintain the osmotic pressure of the nutrient solution.

The replacement of half the external Na^+ with an osmotically equivalent amount of sucrose was without effect on the tension developed in response to ACh. Replacement of all but 22 mM Na^+ with sucrose resulted in a slow decline in the tension developed to a constant dose of ACh. The results varied slightly in the eight diaphragms tested, but in general the tension developed in response to ACh declined to between 25–50% of the control value over a period of 60–150 min. The rise time was unchanged, but there was a gradual increase in the time to half relaxation, until at 60–70 min the ACh response had become a persistent contracture. These findings are illustrated in Fig. 1. It was noteworthy that in one experiment there was a gradual recovery of tension between 60 and 100 min, until the response was of nearly normal force.

Replacement of all the external Na^+ with sucrose, using Tris as a buffering agent, accelerated the changes seen in 22 mM Na^+ (Fig. 1). In six experiments the ACh response started to decline after 10 min, and levelled off at approximately 25% of the control value at 30–40 min. It was still possible to obtain an ACh contraction after 120 min in Na^+-free solution, and again one preparation showed a partial restitution of contractile force after 60 min exposure. The rate of rise fell off somewhat after 70 min in Na^+-free solution, and the duration of the response increased until it equalled the time of exposure of the preparation to ACh. Washing the preparation with normal solution for 10–12 min returned the duration of contraction to less than normal; further, the tension developed was from 140–170% of the control value. These parameters returned to control values over a period of 30–40 min.

It is possible that the partial restoration of tension which was occasionally seen after prolonged exposure to low Na^+ or Na^+-free solution may be related to the partial dependence of the ACh contraction on the Na^+ gradient across the membrane. The initial loss of extracellular Na^+ would reduce this gradient, which would tend to be restored by a later loss of intracellular Na^+ (Simon, Shaw, Bennett & Muller, 1957). Some evidence for this hypothesis is derived from the observation that the restitution of extracellular Na^+ brought about an initial increase in the ACh response to

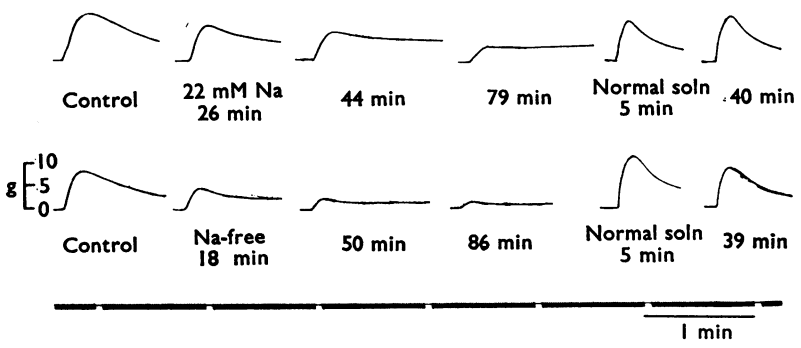

FIG. 1. Effect of Na^+ depletion on the ACh contraction of denervated diaphragm. The top row shows a control contraction (ACh 10^{-5}M), followed by contractions elicited in the presence of 22 mM Na^+, sucrose solution. The second row shows a control contraction by the same ACh concentration, followed by contractions elicited in the presence of Na^+-free sucrose solution. Return to the normal solution temporarily potentiates the contractile force above the control level. In each instance ACh was washed out at the conclusion of the tracing.

greater than normal levels; one would expect the Na^+ gradient to be temporarily increased.

Exposure of a preparation to a Na^+-free sucrose solution which was also Ca^{++}-free resulted in a marked slowing of the rate of rise of the contraction and a reduction in tension over a period of 20 min. By 40 min the ACh response was abolished. The ineffectiveness of ACh in solutions lacking both Na^+ and Ca^{++} recalls the similar findings of Pappano & Volle (1966), who used the perfused superior cervical ganglion of the cat.

As would be expected, transmission was blocked by Na^+-free solutions in contralateral, innervated preparations in 10–15 min.

Sodium substitution with Tris

Experiments were carried out in which an attempt was made to maintain the ionic strength of the bathing solution by substituting all the Na^+ with Tris hydrochloride. It was found that the response to ACh declined to 20–40% of the control value over a period of 20 min (four experiments). Rise time and duration were also shortened. These effects may have been partly due to Ca^{++} chelation by Tris (Mahler, 1961), so the Ca^{++} concentration of the solution was then raised to 4·5 mM ($\times 3$ normal). Tension developed was but slightly improved by the Ca^{++} increase, but the response lengthened until it equalled the time of exposure of the preparation to ACh.

Sodium substitution with lithium

Since Li^+ can replace Na^+ in maintaining the action potential of nerve and muscle (Gallego & Lorente de Nó, 1951), experiments were carried out in which all the external Na^+ was substituted with Li^+. Tris hydrochloride was used as buffer.

The rate of rise and relaxation time were not altered by Li^+ solution. However, tension development fell off slowly over a 60 min period until it was 30–40% of the control value. Restoring the normal Na^+ solution brought back tension development to the control level in 10 min. The response to ACh in Li^+ solution was further depressed by increasing the external Ca^{++} level to 4·5 mM; in contrast to the findings in Tris and sucrose solutions, Ca^{++} increase did not prolong the contraction.

Additional experiments were carried out with Li^+ solutions without Ca^{++}. Here it was found that the ACh response declined to zero in 50 min. Restoring the Ca^{++} to normal (1·5 mM) at this point restored the ACh response to 60% of the control value.

Neuromuscular transmission in the contralateral innervated preparation was blocked completely in 6–8 min by Li^+ solution (Onadera & Yamakawa, 1966; Freeman, 1968). If the response of the postsynaptic membrane is reduced by Li^+ in a quantitatively similar way to that of the ACh receptor of denervated muscle, then the greater efficacy of Li^+ in blocking transmission must be related to a reduction in the presynaptic output of ACh.

Divalent cation changes

Reduction of the external Ca^{++} or Mg^{++} concentration to 0·15 mM and 0·1 mM respectively ($\times 0{\cdot}1$ normal) was without significant effect on the ACh-induced con-

traction. An increase in the Mg^{++} concentration to 5 mM for 10 min before administration of ACh reduced the tension developed to 50–60% of the control value, but did not alter the time course of the contraction. A threefold increase in Ca^{++} reduced the response to approximately 70% of the control value, and increased the relaxation time five-fold (see Fig. 3). A further increase in Ca^{++} to 7·5 mM converted the ACh response to a sustained contracture.

The decreased tension developed in high Ca^{++} or Mg^{++} solutions may reflect the membrane stabilizing effect of these ions, and recalls the results of Takeuchi (1963), who determined the effects of Ca^{++} and Mg^{++} on the ACh-induced conductance change of the endplate membrane. The prolongation of the contraction by raised Ca^{++} may be a function of the ability of the sarcoplasmic reticulum to re-sequester Ca^{++} in the face of continuing depolarization and an increased Ca^{++} gradient.

Effect of $MnCl_2$ on ACh response

It has been shown that the action potentials of crustacean muscles are due to an increase in conductance for divalent cations rather than for Na^+ (Hagiwara & Nakajima, 1966); it is likely that the rising phase of the action potential in vertebrate smooth muscle is also unrelated to an increased Na^+ conductance (Kuriyama, Osa & Toida, 1966). It appeared possible, therefore, that an increase in Ca^{++} conductance could be of major importance in ACh depolarization of denervated muscle. Manganese ions have been shown to suppress such "Ca^{++} spikes" (for references, see Hashimoto & Holman, 1967); consequently their effect on the ACh-induced contraction was determined.

It was found that 1 mM $MnCl_2$ added to a Tris-buffered Na^+ containing solution completely blocked the innervated diaphragm to both direct and indirect stimulation in 6 min. It was possible to break through the muscle membrane blockade by increasing the stimulating voltage from a normal value of 6–8 V for supramaximal stimulation to approximately 100 V.

This level of $MnCl_2$ was completely without effect on the ACh response of the denervated diaphragm, even after 30 min exposure to Mn^{++}. Increasing the Mn^{++} concentration to 4 mM reversibly depressed the ACh response. The dose-response curve in the presence of 4 mM Mn^{++} was parallel to the control, and the dose ratio was approximately 2.

Increasing the external Ca^{++} level to 4·5 mM completely relieved the blockade of the innervated preparation; all parameters returned to normal. However, a similar increase in Ca^{++} concentration did not affect the partial inhibition of the ACh response in the denervated preparation. Further, the effect of Mn^{++} on this response was not altered by concurrently reducing the Ca^{++} level to 0·15 mM, although the time to half relaxation was somewhat shortened in the low Ca^{++} solution.

Thus Mn^{++} appears to block the innervated preparation by processes involving competition with Ca^{++}; its inhibitory effect on the ACh response of the denervated preparation requires a higher concentration of Mn^{++} and cannot be modified by changes in the external Ca^{++} level.

Drug effects on ACh response

Tetrodotoxin and saxitoxin

Katz & Miledi (1966) noted that tetrodotoxin, which appears to inhibit specifically the inward Na^+ current during the action potential (Kao, 1966), was without effect on the electrical properties of the neuromuscular junction. This appears also to be so in the axo-axonic giant synapse of the stellate ganglion of the squid (Katz & Miledi, 1967). Saxitoxin is pharmacologically very similar to tetrodotoxin.

The effects of these drugs have been compared in both the innervated and denervated diaphragm preparations. It was found that tetrodotoxin at a concentration of 10^{-7} g/ml. completely blocked the innervated diaphragm to direct and indirect stimulation in 3 min ; approximately twice as much saxitoxin was required to block this preparation in the same time. These drug levels were totally without effect on the ACh contraction of the denervated diaphragm, even after exposure for 60 min. Additional experiments were carried out in which tetrodotoxin (10^{-7} g/ml.) was added to solutions in which all the Na^+ had been replaced with sucrose, lithium or Tris. Here again the contraction was not modified by the presence of tetrodotoxin.

Procaine hydrochloride

In distinction to tetrodotoxin and saxitoxin, procaine hydrochloride (PrHCl) was found to block reversibly the ACh response of the denervated diaphragm ; dose-response curves for ACh were shifted to higher concentrations. Figure 2 shows the effect of two concentrations of PrHCl on the ACh dose-response curve. As the control curves differed somewhat in slope the data could not be pooled ; the results

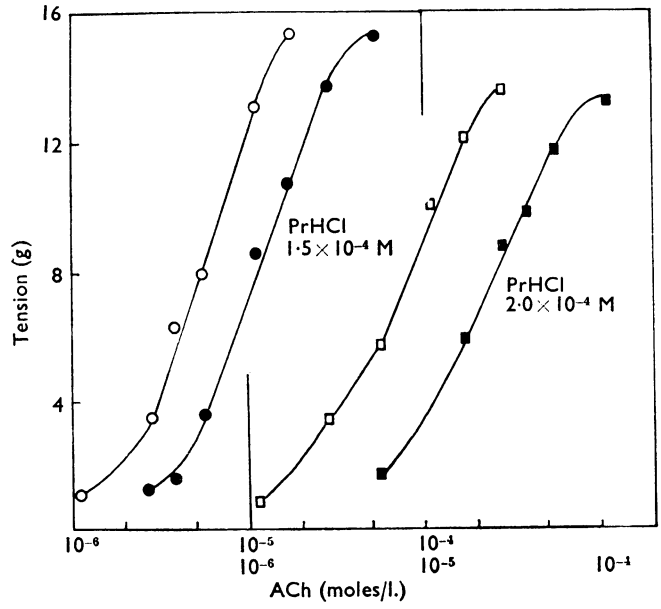

FIG. 2. Effect of procaine hydrochloride on the ACh dose-response curve. Two levels of PrHCl are shown. The graph has been divided to show two experiments ; the upper figures on the abscissa refer to the left hand segment, the lower to the right hand segment.

of two typical experiments are shown in Fig. 2. Using probit analysis the difference in slope in each case was shown not to be significant at the 5% probability level. Further it was possible to equal the maximum ACh contraction in the presence of PrHCl by raising the ACh level. Higher concentrations of PrHCl were tested in order to extend the data ; it was found, however, that at 3 or 4×10^{-4}M it was impossible to obtain a steady response to ACh. The data illustrated in Fig. 2 were obtained by adding ACh to the organ bath after 10 min exposure to PrHCl ; the preparation was then washed for 5 min and PrHCl added for 10 min before the next ACh dose. This procedure did not produce consistent responses with higher levels of PrHCl and one must assume that a sufficient quantity of the drug had entered the cell to interfere with Ca^{++} release by the reticulum (Bianchi, 1968).

In order to test the apparently competitive nature of the PrHCl inhibition over the limited concentration range available, we carried out the dose-ratio test described by Paton & Rang (1965). Tubocurarine was used as the second ACh antagonist. The finding of Elmqvist & Thesleff (1960) that TC competitively inhibits the ACh response of the denervated diaphragm was confirmed. Dose response-curves were drawn, and were found to be parallel over the range of concentrations of TC shown in Table 2.

The dose ratio (DR) is defined as D_1/D where D_1 is the dose of an agonist in the presence of an inhibitor, required to produce the same response as the concentration of the agonist, D, in the absence of inhibitor. If two antagonists giving dose ratios DR_1 and DR_2 are both competitive inhibitors of the agonist (ACh) the dose ratio obtained in the presence of both inhibitors (DR_{1+2}) should equal $DR_1 + DR_2 - 1$. If the two inhibitors do not compete for the same active site on the receptor $DR_{1+2} = DR_1.DR_2$.

Experiments to test the nature of PrHCl inhibition were carried out in the following manner. First, the ACh dose-response curve for each preparation was determined. The dose ratios for TC and PrHCl were then determined separately, and then the combined dose ratio DR_{1+2} was determined. A difficulty was encountered in that the combined dose of TC and PrHCl caused a prolonged inhibition of the ACh response which persisted for upwards of an hour in some preparations. Consequently it was found difficult to obtain more than one or two values of DR_{1+2} in each preparation. It was found (see Table 2) that the values obtained were consistent with competitive inhibition, or were slightly too large. In no case was $DR_{1+2} = DR_1.DR_2$. Thus one may conclude that PrHCl is essentially a competitive inhibitor of ACh, but that its action also contains a non-competitive element.

Interactions between Ca^{++}, Mg^{++}, PrHCl and ACh were also studied, in an attempt to separate the membrane effects of PrHCl from those operative at the reticular level (Bianchi, 1968). Thus Ca^{++} and Mg^{++} may be expected to have similar effects on the membrane (Takeuchi, 1963) but Mg^{++} cannot substitute for

TABLE 2. *Dose ratio test for ACh antagonism by TC and procaine*

TC (M)	Procaine (M)	DR_{1+2}	$DR_1 + DR_2 - 1$	$DR_1.DR_2$
10^{-7}	$1{\cdot}5 \times 10^{-4}$	2·9	3·2	4·4
10^{-7}	$1{\cdot}5 \times 10^{-4}$	4·4	4·0	6·2
2×10^{-7}	$1{\cdot}5 \times 10^{-4}$	6·7	4·8	7·9
3×10^{-7}	$1{\cdot}5 \times 10^{-4}$	18·5	11·0	32·0
4×10^{-7}	$1{\cdot}5 \times 10^{-4}$	5·0	6·4	8·9

Ca^{++} in excitation-contraction (E-C) coupling. The results of typical experiments are shown in Fig. 3. As was noted earlier, low Ca^{++} or Mg^{++} solutions did not affect the ACh response. The inhibitory response to PrHCl ($1{\cdot}5 \times 10^{-4}$M) was diminished by concurrently lowering either the Ca^{++} or Mg^{++} concentration. This effect was particularly marked in 0·15 mM Ca^{++}. Increasing the level of divalent cation increased the inhibitory power of PrHCl. Mg^{++} (5 mM) did not affect the time course of the contraction; 4·5 mM Ca^{++} both reduced the amplitude and prolonged the ACh contraction. Thus both PrHCl and divalent cations appear to stabilize the membrane and their effects are synergistic.

PrHCl was found to block neuromuscular transmission in contralateral innervated preparations at approximately 5×10^{-4}M. It was noteworthy that the blockade could be relieved by increasing the Ca^{++} level of the solution to 4·5 mM. Relief was never complete; twitch tension was restored to 50–70% of the control level. Presumably the reduction in amplitude of the nerve action potential by procaine inhibited ACh release. This effect was overcome by raising the Ca^{++} level. Post-synaptic inhibition by procaine may well have been potentiated by raised Ca^{++}.

Effect of thiocyanate on ACh response

If one assumes that PrHCl acts at the level of the ACh receptor to block the initial excitatory event in the E-C coupling sequence then it is of interest to determine interactions between ACh and events occurring more distally in the E-C sequence. SCN^- is the most active of a lyotropic series of anions which in innervated amphibian muscle are believed to act on the transverse tubular element, to alter the E-C process so as to increase the duration of the active state (Bianchi, 1968; Hodgkin & Horowicz, 1960).

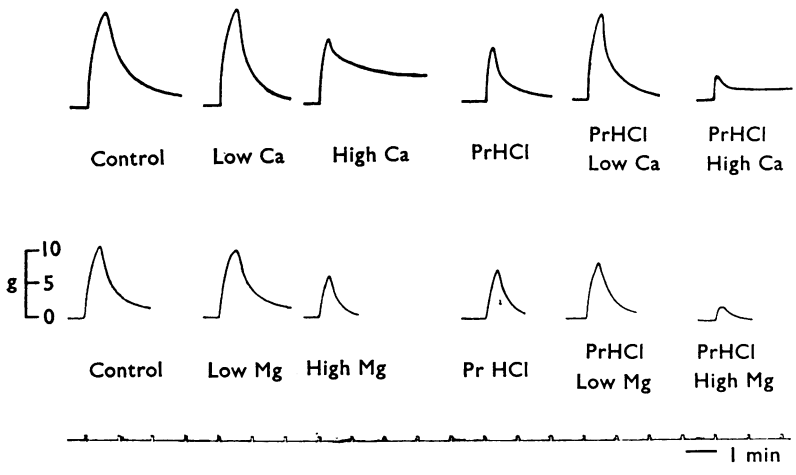

FIG. 3. Effect of divalent cation variation on the inhibition by PrHCl of the ACh response. The upper row shows a control contraction (ACh 10^{-5}M), followed by contractions in the presence of 0·15 mM Ca^{++}, 4·5 mM Ca^{++}, $1{\cdot}5 \times 10^{-4}$M PrHCl, PrHCl + 0·15 mM Ca^{++}, and PrHCl + 4·5 mM Ca^{++}. The lower row shows a control contraction followed by contractions in the presence of 0·1 mM Mg^{++}, 5 mM Mg^{++}, $1{\cdot}5 \times 10^{-4}$M PrHCl, PrHCl + 0·1 mM Mg^{++}, and PrHCl + 5 mM Mg^{++}. In each instance the drugs were washed out at the end of each tracing.

Experiments were carried out to determine the effect of replacing 12 mM Cl^- in the bathing solution with SCN^-. The ACh response was both potentiated and prolonged. The effect of SCN^- on the ACh dose-response curve is illustrated in Fig. 4. It may be noted that the curves diverged at high levels of ACh, suggesting that the prolongation of the contraction was sufficient to augment the contraction height

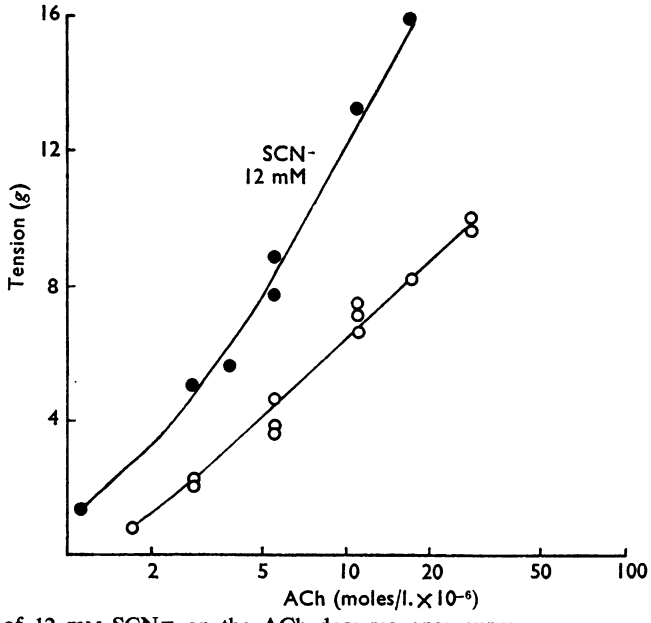

FIG. 4. Effect of 12 mM SCN^- on the ACh dose-response curve.

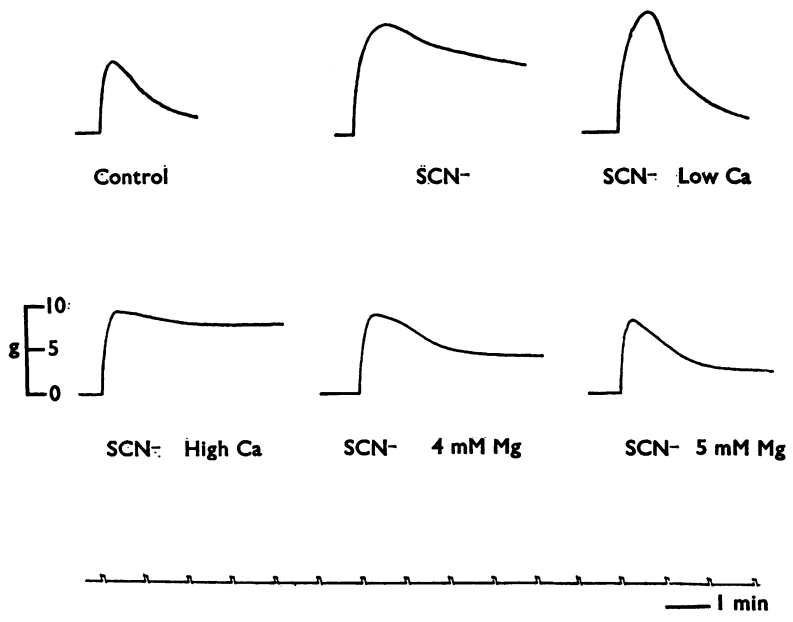

FIG. 5. Effect of divalent cation variation on the SCN^- potentiation of the ACh response. The upper row shows a control response (ACh 10^{-5}M), followed by the potentiated response in 12 mM SCN^-. Low Ca^{++} (0·15 mM) increases the amplitude, 4·5 mM Ca^{++} (lower row) reduces and prolongs the response. High Mg^{++} (4 or 5 mM) reduces the response.

(see above). Both the amplitude and duration of the ACh response in the presence of SCN^- were dependent on the Ca^{++} and Mg^{++} concentration of the external medium. Figure 5 shows that reduction of the external Ca^{++} level to 0·15 mM reduced the duration of the contraction, and slightly increased the amplitude. Increasing the external Ca^{++} level to 4·5 mM reduced the amplitude of the ACh contraction and prolonged it until it equalled the time of exposure to ACh. On the other hand, increasing the external Mg level to 4 or 5 mM reduced the amplitude to that of the control without SCN^-. The contraction was still, however, prolonged by a factor of two compared to the control contraction. Thus the ability of SCN^- to augment and prolong the ACh response can be modified by Ca^{++} and Mg^{++}.

It may be noted that SCN^- augmented but did not prolong the indirectly elicited twitch of the contralateral innervated preparation.

Discussion

The response of the chronically denervated diaphragm to acetylcholine appears in general to resemble that of the postsynaptic membrane. Evidence from studies involving alteration of the external ionic environment confirms the thesis of del Castillo & Katz (1955) that ACh " short-circuits " the excitable membrane, allowing ionic currents to flow according to their gradients. Thus the ACh contraction can be related to the Na^+ gradient across the membrane, although it is not entirely dependent upon the presence of Na^+. It may be that under conditions of almost total Na^+ depletion movement of Ca^{++} is sufficient to initiate contraction. It is of interest that the lengthening of the ACh contraction seen in low Na^+-sucrose solutions did not occur when Tris or Li^+ was substituted for Na^+. Raising the Ca^{++} concentration in Tris solution, as in Na^+ solution, prolonged the response. This prolongation was not seen in Li^+ solution when external Ca^{++} was raised. Thus Li^+ appears to be more effective than Na^+ in maintaining the relaxing mechanism.

The reason for the brevity of the ACh response is obscure. It cannot be related to ACh hydrolysis, because the denervated preparation contains AChE only in the region of the degenerating synapse (Eränkö & Teräväinen 1967). Further, the carbachol response, although longer than the ACh response, is nevertheless of finite length. The observation of Lüllmann & Reis (1967) that the denervated diaphragm relaxes in spite of a continuing depolarization suggests that, as in a K^+-induced contraction, the sarcoplasmic reticulum is able to re-sequester Ca^{++} although the membrane remains depolarized. This observation makes a comparison of the duration of conductance changes at the endplate with the contractile event hazardous.

The involvement of the reticular relaxing system in the ACh contraction means that drug interactions may occur at three possible points: first at the ACh receptor which may be located on the surface membrane, second at the level of the transverse tubule where the membrane event is propagated into the muscle cell, and third at the level of the terminal cisternae, where Ca^{++} is released to initiate contraction.

There is some evidence (Brody, 1966 ; Howell, Fairhurst & Jenden, 1966) that the reticular relaxing system alters following denervation, and has an increased ability to sequester Ca^{++}. Brody (1966) suggested that E-C coupling is facilitated after denervation, and that this may be a factor in producing fibrillations.

The finding that the ACh response is totally unaffected by concentrations of tetrodotoxin or saxitoxin that rapidly block the innervated preparation confirms

the thesis that the permeability changes following ACh-receptor interaction differ from those involved in the generation of the action potential. Whether the tetrodotoxin-sensitive receptor and the ACh receptor exist in series or in parallel is less clear. It is possible that the ACh receptor may develop in response to denervation in the transverse tubular element, and thus occur more distally in the chain of E-C coupling than the tetrodotoxin receptor.

The findings that the ACh receptor is less sensitive to Mn^{++} than the innervated muscle rules out the possibility that the ACh generates a "Ca^{++} spike" such as occurs in smooth and crustacean muscle. The insensitivity of the Mn^{++} inhibition of the ACh receptor to variation in external Ca^{++} suggests that Mn^{++} is not competing with Ca^{++}, but offers no indication as to its site of action.

If it be granted that SCN^- affects the E-C coupling sequence by lowering the threshold for excitation at the level of the transverse tubule system, then the ACh receptor must be located either at this level or more proximal to it. The potentiation of the ACh response by SCN^- was reduced by increased concentrations of either Ca^{++} or Mg^{++}. This may represent stabilization of the ACh receptor, as has been suggested for PrHCl.

It is of interest that Ca^{++}, Mg^{++} and PrHCl synergize in denervated muscle. Other workers have noted antagonism between PrHCl and Ca^{++} in other systems. Thus in smooth muscle (Feinstein, 1966), lobster nerve (Blaustein and Goldman, 1966) or frog spinal ganglia (Aceves and Machne, 1963) Ca^{++} increase relieved local anaesthetic inhibition. These authors attributed their results to competition between PrHCl and Ca^{++} at a membrane site which controls the increase in Na^+ conductance upon electrical stimulation. Our finding again emphasizes the separateness of the membrane permeability changes following ACh, and the changes associated with electric excitation.

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REFERENCES

ACEVES, J., & MACHNE, XENIA (1963). The action of calcium and of local anaesthetics on nerve cells, and their interaction during excitation. *J. Pharmac. exp. Ther.*, **140**, 138–148.

AXELSSON, J., & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol., Lond.*, **149**, 178–193.

BERÁNEK, R., & VYSKOČIL, F. (1967). The action of tubocurarine and atropine on the normal and denervated rat diaphragm. *J. Physiol. Lond.*, **188**, 53–66.

BIANCHI, C. P. (1968). Pharmacological actions on excitation-contraction coupling in striated muscle. *Fedn Proc.*, **27**, 126–131.

BLAUSTEIN, M. P., & GOLDMAN, D. E. (1966). Competitive action of calcium and procaine on lobster axon. *J. gen. Physiol.*, **49**, 1043–1064.

BRODY, I. A. (1966). Relaxing factor in denervated muscle: a possible explanation for fibrillations. *Am. J. Physiol.*, **211**, 1277–1280.

CASTILLO, J. DEL, & KATZ, B., (1954). The membrane change produced by the neuromuscular transmitter. *J. Physiol. Lond.*, **125**, 546–565.

CASTILLO, J. DEL, & KATZ, B. (1955). Local activity at a depolarized nerve-muscle junction. *J. Physiol., Lond.*, **128**, 396–411.

ELMQVIST, D., & THESLEFF, S. (1960). A study of acetylcholine induced contractures in denervated mammalian muscle. *Acta Pharmac. tox.*, **15**, 84–93.

ERÄNKÖ, O. & TERÄVÄINEN, H. (1967). Cholinesterases and eserine-resistant carboxylic esterases in degenerating and regenerating motor end plates of the rat. *J. Neurochem.*, **14**, 947–954.

FEINSTEIN, M. B. (1966). Inhibition of contraction and calcium exchangeability in rat uterus by local anaesthetics. *J. Pharmac. exp. Ther.*, **152**, 516–524.

FREEMAN, SHIRLEY E. (1968). Ionic influences on succinylcholine blockade of the mammalian neuromuscular junction. *Br. J. Pharmac. Chemother.*, **32**, 546–566.

GALLEGO, A., & LORENTE DE NÓ, R. (1951). On the effect of ammonium and lithium ions upon frog nerve deprived of sodium. *J. gen. Physiol.*, **35**, 227–244.

HAGIWARA, S., & NAKAJIMA, S. (1966). Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine and manganese ions. *J. gen. Physiol.*, **49**, 793–818.

HASHIMOTO, Y. & HOLMAN, MOLLIE E. (1967). Effect of manganese ions on the electrical activity of mouse vas deferens. *Aust. J. exp. Biol. med. Sci.*, **45**, 533–539.

HODGKIN, A. L., & HOROWICZ, P. (1960). The effect of nitrate and other anions on the mechanical response of single muscle fibres. *J. Physiol., Lond.*, **153**, 404–411.

HOWELL, J. N., FAIRHURST, A. S., & JENDEN, D. J. (1966). Alterations of the calcium accumulating ability of striated muscle following denervation. *Life Sci., Oxford*, **5**, 439–446.

JENKINSON, D. H., & NICHOLLS, J. G. (1961). Contractures and permeability changes produced by acetylcholine in depolarized muscle. *J. Physiol., Lond.*, **159**, 111–127.

KAO, C. Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18**, 997–1050.

KATZ, B., & MILEDI, R. (1966). The production of endplate potentials in muscles paralyzed by tetrodotoxin. *J. Physiol., Lond.*, **185**, 5P–6P.

KATZ, B., & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol., Lond.*, **192**, 407–436.

KRNJEVIĆ, K., & MITCHELL, J. F. (1960). Diffusion of acetylcholine in agar gels and in the isolated rat diaphragm. *J. Physiol., Lond.*, **153**, 562–572.

KURIYAMA, H., OSA, T., & TOIDA, N. (1966). Effect of tetrodotoxin on smooth muscle cells of the guinea-pig taenia coli. *Br. J. Pharmac. Chemother.*, **27**, 366–376.

LETLEY, E. (1960). The effect of temperature on the direct muscle twitch response and the action of drugs on the isolated denervated rat diaphragm. *Br. J. Pharmac. Chemother.*, **15**, 345–350.

LOOMIS, T. A., & KONKER, A. C. (1967). Effects of an organic phosphate on the response of denervated muscle to acetylcholine and some neuromuscular blocking agents. *Archs int. Pharmacodyn. Thér.*, **165**, 308–318.

LÜLLMANN, H., & REIS, E. (1967). The relationship between the membrane potential and K^+ or acetylcholine contraction in the chronically denervated diaphragm of the rat. *Pflügers Arch. ges. Physiol.*, **294**, 113–118.

MAHLER, H. R. (1961). The use of amine buffers in studies with enzymes. *Ann. N.Y. Acad. Sci.*, **92**, 426–439.

MCINTYRE, A. R., & KING, R. E. (1943). Contraction of denervated muscle produced by d-tubocurarine. *Science, N.Y.*, **97**, 516.

ONADERA, K., & YAMAKAWA, K. (1966). The effects of lithium on the neuromuscular junction of the frog. *Jap. J. Physiol.*, **16**, 541–550.

PAPPANO, A. J., & VOLLE, R. L. (1966). Observations on the role of calcium ions in ganglionic responses to acetylcholine. *J. Pharmac. exp. Ther.*, **152**, 171–180.

PATON, W. D. M., & RANG, H. P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc. B.*, **163**, 1–44.

SIMON, SHIRLEY E., SHAW, F. H., BENNETT, S., & MULLER, M. (1957). The relationship between sodium, potassium and chloride in amphibian muscle. *J. gen. Physiol.*, **40**, 753–777.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the endplate membrane during the action of transmitter. *J. Physiol., Lond.*, **167**, 141–155.

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