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MECHANISM OF THE PARALYSING ACTION OF PIPERAZINE ON ASCARIS MUSCLE

BY

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The effects of piperazine on *Ascaris* muscle cells have been investigated with electrophysiological techniques. These cells have an average resting potential of about 30 mV interrupted by rhythmic spikes of myogenic origin (1 to 7 spikes/sec). With piperazine (10^{-3}, w/v), the average resting potential increases above 40 mV and the pacemaker activity is suppressed. These changes are similar to those temporarily produced in the same cells by the electrical stimulation of inhibitory nerve fibres. Electrophoretic application of piperazine to different areas of the muscle cells shows that this drug hyperpolarizes their membrane only when applied to the region where both excitatory and inhibitory neuromuscular synapses are located. The degree of muscle hyperpolarization induced by piperazine depends upon the extracellular chloride concentration, decreasing when a fraction of the chloride ions is replaced by the larger, supposedly nonpenetrating, sulphate anions. Piperazine may, therefore, be regarded as a pharmacological analogue of a natural inhibitory transmitter.

Soon after the introduction of piperazine as an anthelmintic, Goodwin & Standen (1954) noticed that worms expelled after treatment with this compound were narcotized, motionless and relaxed, but not killed ; tone and movement reappeared upon immersion in Ringer solution at 37° C. This result was confirmed *in vitro* by Poynter (1955), using piperazine adipate against *Parascaris equorum*, and by Standen (1955), who tested the effect of several piperazine salts against *Ascaris lumbricoides* from the pig and found that worms placed in a solution (1 : 200 to 560) of any of the salts were narcotized but recovered in less than 3 hr in a drug-free medium. Recovery was complete even after 48 hr exposure to piperazine at these concentrations.

Working with fragments of *Ascaris*, Norton & DeBeer (1957) showed that the stimulating action of acetylcholine was blocked by piperazine as well as by D-tubocurarine. It was concluded that the paralysing action of piperazine was due to a curare-like effect on the neuromuscular junctions.

Recent work on the electrical activity of the somatic muscle cells of *Ascaris* (DeBell, del Castillo, & Sanchez, 1963 ; del Castillo, de Mello & Morales, 1963), casts doubt on the above hypothesis. The contraction of *Ascaris* muscle is not initiated by nerve impulses transmitted in a one-to-one fashion across neuromuscular

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junctions but by rhythmic spike potentials generated by pacemakers in the muscle membrane itself. Acetylcholine depolarizes the muscle cells, thereby increasing the frequency of the spike potentials and the degree of contraction. Although these effects are blocked by D-tubocurarine, the latter does not prevent the generation of spontaneous muscle spike potentials even at concentrations as high as 10^{-3} (w/v).

The action of piperazine on *Ascaris* muscle has been reinvestigated using the techniques and preparations which were employed in the work just described. The results obtained, briefly reported in two preliminary notes (del Castillo, Morales & Sanchez, 1963 ; de Mello, del Castillo & Morales, 1963), suggest that this drug acts as a pharmacological analogue of a natural inhibitory neurohormone, increasing the resting potential of the muscle membrane above the range in which pacemaker activity is possible.

METHODS

Preparations. The body wall of *Ascaris lumbricoides*, var. *suum*, obtained from a local slaughter house, was always used. A cylindrical fragment of the worm, about 2 to 3 cm long, was split open along one of the lateral lines. After removal of the intestine, the resulting rectangular piece of tissue was pinned (cuticle side down) on a slab of Teflon inside a double-walled Perspex chamber.

Solutions. Ascaris tissues can be maintained in a functional condition immersed in an isotonic salt solution made by diluting 30 parts of sea water with 70 parts of distilled water (Hobson, Stephenson & Beadle, 1952). Although the ionic composition of this solution differs from that of the perienteric fluid of *Ascaris*, particularly with regard to the nature of the anions, it has been found suitable for electrophysiological work (Jarman, 1959 ; DeBell *et al.*, 1963).

In the experiments involving changes in the ionic composition of the bathing solution, artificial sea water was employed. This was prepared by mixing in appropriate proportions isotonic stock solutions of the different salts (Hodgkin & Katz, 1949). When the mixture was diluted to 30% (v/v), a saline solution was obtained with the following ionic concentrations (mM): Na^+ 135 ; K^+ 3 ; Ca^{++} 3 ; Mg^{++} 15.7 ; Cl^- 175.4 ; HCO_3^- 0.8. Solutions with altered concentrations of potassium and chloride were also prepared. In some, potassium was left out or increased at the expense of sodium. In others, the concentration of chloride ions was reduced by replacing a fraction of the sodium chloride stock with isosmotic amounts of sodium sulphate. The presence of sufficent ionized calcium in the high sulphate concentrations was ensured by the addition of calcium sulphate (8 mM).

Electrical techniques. Resting and spike potentials were recorded from the muscle cell bellies with conventional intracellular microelectrodes. Spike potentials and muscle inhibitory potentials were elicited in some experiments by direct electrical stimulation of the nerve cord region with the aid of bipolar electrodes made of platinum wire (0.5 mm in diameter). Piperazine was also applied to localized regions of the muscle cell surface using the electrophoretic technique described by Nastuk (1951).

Drugs. Piperazine citrate was used in most experiments ; however, tests were also done with piperazine hexahydrate and sodium citrate alone. Stock solutions of these compounds were prepared in 30% sea water, adjusted to a *p*H of 7 and added to the bathing solutions in sufficient amounts to give the final concentrations indicated below. Concentrations of these compounds are given as w/v.

Temperature. All the experiments were performed with the bathing solution maintained at between 39 to 40° C by circulating warm water between the walls of the chamber.

RESULTS

Changes in muscle membrane potential. Resting and spike potentials were recorded from at least twenty cell bellies while the preparation was immersed in 30% sea water. Piperazine was then added to give a final concentration between 10^{-5} and 10^{-2}. After at least 15 min had been allowed for equilibration, an equal number of cells were impaled. The average resting potential was now increased, and if the piperazine concentration was 10^{-4} or higher, the spontaneous spike potentials disappeared.

The magnitude of the hyperpolarization depended upon the concentrations of the drug in the bathing solution, as shown by experiments in which the average membrane potential of the muscle cells was determined in preparations equilibrated with increasing concentrations of piperazine. Fig. 1 shows that, at a concentration of about 10^{-5} to 10^{-3}, the potential across the muscle cell membrane increased with a slope of about 8 mV per tenfold increase in the piperazine concentration.

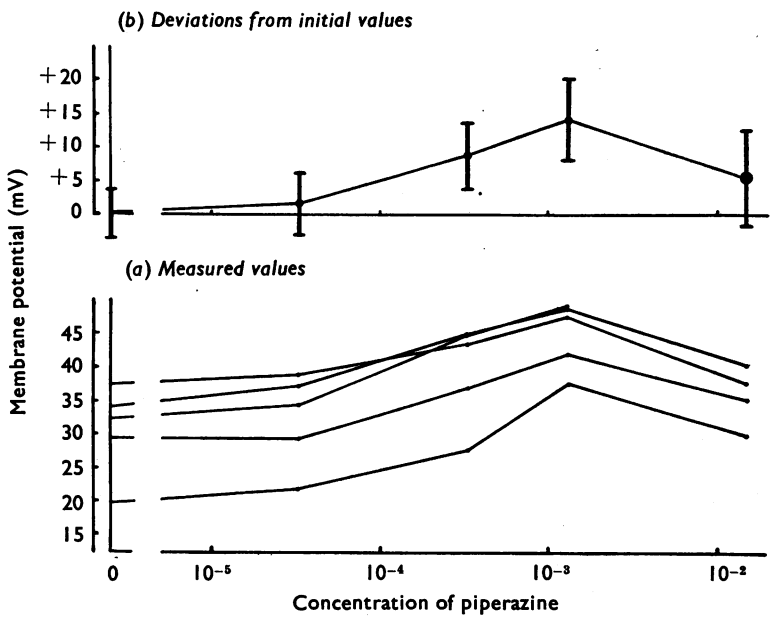

Fig. 1. Influence of piperazine citrate on the membrane potential of *Ascaris* muscle cells (medial bellies). (*a*) shows the change in membrane potential (ordinate, mV) with increasing concentrations of piperazine citrate (abscissa, w/v) in five different preparations. Each point gives the average of twenty measurements taken from different cells. Although the initial average membrane potentials in 30% sea water varied from 19 to 37 mV, the five curves show a relatively uniform change. The highest piperazine concentration was not tested in one of the preparations. The points in (*b*) give the averages of all the measurements in the five preparations, expressed as the difference between the observed values at each concentration and the average resting potential in 30% sea water in the absence of piperazine. The standard errors of the means were from about 0.4 to 0.8 mV; therefore they did not exceed the radius of the solid circle drawn around the point corresponding to the highest piperazine concentration. Twice the total standard deviation of each of the 100 measurements (80 only for the 1.5×10^{-2} concentration) is shown by a vertical bar.

Suppression of rhythmic spike activity occurred in *Ascaris* preparations when the muscle resting potential increased above about 40 mV. Although in the averaged results of our experiments this level was reached in a piperazine concentration of just over 3.3×10^{-4}, the exact amount of the drug needed to block spike activity and, in consequence, completely to paralyse the muscle, depended upon the initial average resting potential of the preparation in 30% sea water.

When the piperazine concentration was increased to 10^{-2} the average membrane potential fell to below the values found at a concentration of 10^{-3}. The results described in the next section suggest that this phenomenon may be due to a diffuse nonspecific, depolarizing effect of the drug upon the entire surface of the muscle cell.

In agreement with previous observations (see Introduction) the membrane hyperpolarization and block of spike activity induced by piperazine, at least by concentrations up to 10^{-3}, were completely reversible. The membrane potential decreased and the spikes reappeared when the preparations were washed with fresh diluted sea water.

Similar experiments were performed to see whether citrate by itself, or the decreased concentration of calcium ions caused by it, might be responsible for any of the changes described. These experiments gave negative results. *Ascaris* muscle cells proved to be rather insensitive to low concentrations of calcium ions. Sodium citrate, in concentrations as high as 10^{-3}, failed to produce significant changes in either resting potential or spike activity. In one preparation, for instance, the resting potential determined in thirty bellies was of 35.4 ± 1.0 mV (mean and standard error). After immersion of the preparation in a 10^{-3} concentration of sodium citrate, the mean resting potential of another thirty impaled bellies was 33.7 ± 1.0 mV.

Experiments in which piperazine hexahydrate was added to the bathing solution gave essentially the same results as those obtained with piperazine citrate.

Site of action of piperazine. Experiments in which acetylcholine was applied to localized areas of the surface of *Ascaris* muscle cells with a micropipette showed that receptors for this compound are not distributed over the entire membrane but restricted to the region of the muscle syncytium (del Castillo *et al.*, 1963a). In consequence, the depolarization elicited by a given acetylcholine concentration tends to be greater in the bellies close to the nerve cord (medial bellies) than in those situated near the lateral line (lateral bellies); the latter are joined to the syncytium by longer arms, in which nonconducted potential changes suffer a larger electrotonic attenuation.

In a similar fashion, the hyperpolarization induced by piperazine was seen to be greater in medial than in lateral bellies. This was confirmed by the experiments summarized in Table 1, showing the changes in resting potential elicited by the same concentrations of piperazine in medial and lateral bellies of four different preparations. To minimize systematic errors such as those due to sudden changes in the properties of the tip of the microelectrode, medial and lateral bellies were impaled alternately. These experiments demonstrate that the average hyperpolarization induced by a given concentration of piperazine is about 2.5-times larger in medial than in lateral bellies.

TABLE 1

HYPERPOLARIZATION PRODUCED BY PIPERAZINE ON MEDIAL AND LATERAL ASCARIS MUSCLE CELLS

Resting potentials in mV are given as means and standard errors for groups of twenty cells at 39 to 40° C

Expt.	Piperazine concentration	Medial bellies			Lateral bellies		
		Control	Piperazine	Difference	Control	Piperazine	Difference
1	10^{-3}	28·3±0·98	41·1±1·17	12·8	33·3±1·48	38·3±1·36	5·0
2	$3{\cdot}3\times10^{-4}$	27·2±0·50	34·9±1·11	7·7	30·5±1·04	34·7±1·88	4·2
3	$6{\cdot}6\times10^{-4}$	29·5±1·06	40·1±1·02	10·6	35·0±1·01	38·7±1·40	3·7
4	$6{\cdot}6\times10^{-4}$	30·7±0·79	41·9±1·06	11·2	35·3±1·13	39·4±1·26	4·1
Mean		28·9	39·5	10·6	33·5	37·8	4·3

In other experiments, piperazine was applied electrophoretically to various areas of the muscle cell surface with the aid of a micropipette filled with a concentrated solution of this compound ; the distribution of the piperazine receptors was found to be similar to that of acetylcholine receptors. No changes in the membrane potential could be observed when piperazine was applied to the spindles, bellies and the accessible region of the arms. However, slow hyperpolarizations, lasting for 30 sec or more, were always produced when the drug was delivered in the vicinity of the nerve cord.

Although the time course of these hyperpolarizations was not investigated, their long duration suggests that the piperazine receptors are located in sites shielded from the bulk of the extracellular solution by effective diffusion barriers. A likely place is the surface of the muscle syncytium in contact with the nerve cord, where the synapses between nerve cord fibres and the muscle membrane are established (Rosenbluth, 1964).

In an attempt to find out whether the lack of action of piperazine applied to the surface of the bellies and spindles might be due to the small size of the drug-filled microelectrodes (outside diameter of the tip about 1 μ) which might prevent the attainment of sufficiently high piperazine concentrations at the cell membrane, some of the pipettes were broken, so that the diameter of the tip was increased to about 20 μ. When these tips, from which piperazine diffused freely, were momentarily brought near the surface of the bellies, membrane *depolarizations,* instead of an increase in the resting potential, were observed. It is likely, therefore, that the reduction in the average membrane potential observed in preparations exposed to piperazine concentrations of 10^{-2} was due to changes taking place in the entire cell surface, in contrast to the specific and localized effects produced by lower concentrations of the drug.

Spontaneous spike potentials. In a number of experiments the effects of adding piperazine to the bathing solution were followed with the microelectrode continuously inserted into a cell belly. In this way changes in resting potential and spike activity can be followed as a function of time. Fig. 2 illustrates such records, showing how both the amplitude and the frequency of the rhythmic spontaneous potentials decreases as the membrane potential increases. These changes should be expected, since the rate of the rhythmic spike potentials has been shown to be inversely related

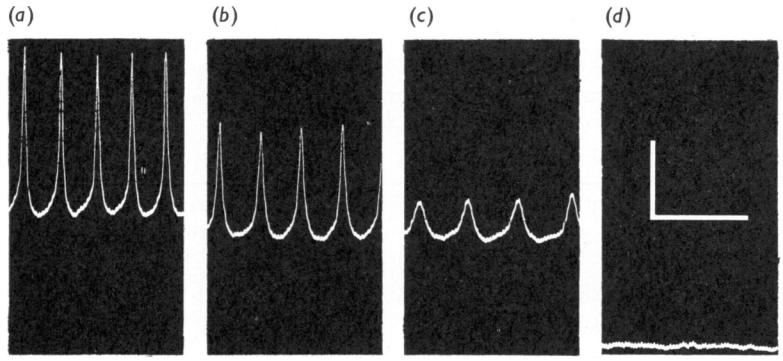

Fig. 2. Changes in the resting and spike potentials of an *Ascaris* muscle cell following the addition of piperazine to the bathing solution. (*a*) shows spike activity in 30% sea water; resting potential, 29 mV; frequency about 5.4 spikes/sec. Between (*a*) and (*b*), piperazine was added to a concentration of 6.6×10^{-4}; the resting potential increased while both frequency and amplitude of the spikes decreased. In (*b*) the resting potential was 32 mV and the frequency decreased to 4.7 spikes/sec; in (*c*) they were 33 mV and 3.7 spikes/sec respectively. The rhythmic potential changes disappeared soon after (*c*) ,while the resting potential increased further. (*d*) shows maximal cell hyperpolarization, with a membrane potential of about 46 mV. Calibrations in (*d*): vertical, 10 mV; horizontal, 0.5 sec.

to the membrane potential at the syncytial region, disappearing when it increases above 40 mV (del Castillo *et al.*, 1963a).

It was impossible, however, to establish a unique correlation between the amplitude and the frequency of the spike potentials on the basis of single cell experiments. Upon the addition of piperazine, these two variables decreased with seemingly independent time courses which varied from cell to cell. This is not really surprising, since the frequency of the spikes is determined, in active preparations, by a single pacemaker. On the contrary, the amplitude of the spikes in any particular cell depends upon the membrane potential prevailing at the region where its arm joins the syncytium. A hyperpolarization at this point can effectively prevent the conduction of spikes into the arm ; this was shown by experiments in which the injection of anodal current into a cell belly blocked the spike activity in the surrounding cells while producing only very small changes in their resting potential (DeBell *et al.*, 1963).

In some cells both frequency and amplitude of the spikes decreased as the resting potential increased (for example, Fig. 2). In others, the amplitude of the spikes decreased gradually until they disappeared completely, without great changes in frequency (Fig. 3). Yet in others the frequency decreased soon after piperazine had been added to the bath and the spikes disappeared suddenly before large changes in amplitude occurred (Fig. 4).

These variable relationships between spike frequency and amplitude suggest that the pacemaker site and the region of attachment of a particular cell arm to the syncytium can be affected by the drug at different rates. In the experiment of Fig. 3, for instance, the spike potentials were blocked before the potential difference across

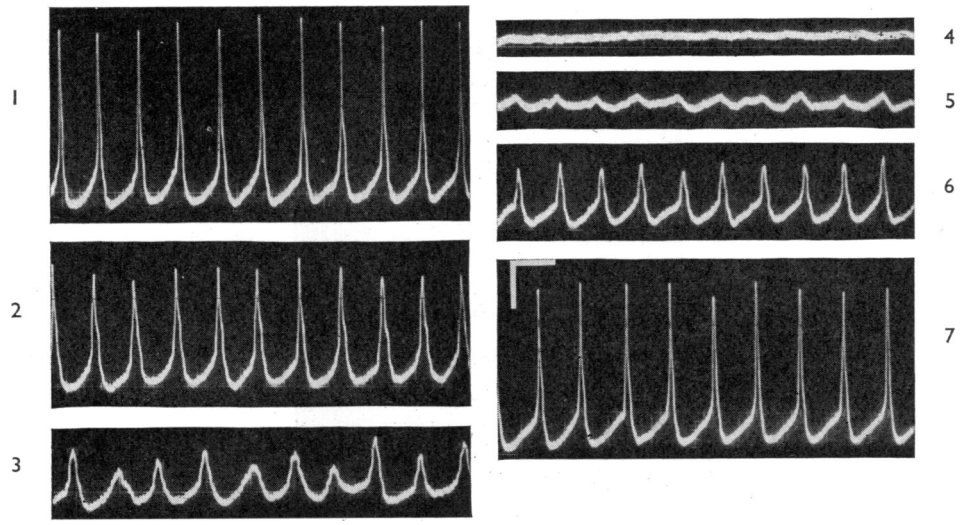

Fig. 3. Suppression of rhythmic spike potentials in an *Ascaris* muscle cell by piperazine, and recovery after removal of the drug. No large changes in spike frequency occurred in this cell. (1) shows spike activity in 30% sea water; resting potential, 35 mV. Between (1) and (2) piperazine was added to the bath solution, in a concentration of 6.6×10^{-4}. Between (4) and (5) the drug was removed from the bath. The maximal membrane potential in record (4) was of about 49 mV. The approximate frequency of spike potentials in records 1 to 7 was: 5.5, 5.4, 5.15, 0, 5.3, 5.3 and 5.15 spikes/sec respectively. Calibrations, in (7): vertical, 5 mV; horizontal, 0.2 sec.

the pacemaker membrane was increased. A reversed situation appears to be responsible for the results illustrated in Fig. 4. The reasons for the different rate of action of the drug on different regions of the muscle syncytium are open to conjecture. A possible explanation is the existence of diffusional barriers of various degrees of effectiveness, an assumption which is supported by some experiments such as that illustrated in Fig. 5. When piperazine was added to the bathing solution the amplitude of the spikes recorded from this cell was reduced without large concomitant changes in frequency, as if the pacemaker was less accessible to the drug than the neural end of the arm. After the action potentials had been fully blocked, the preparation was rinsed with fresh saline. The amplitude of the spike potentials now recovered well before the frequency, suggesting that, at the pacemaker, the piperazine concentration, which was built up slowly, decreased also with a marked delay.

Inhibitory potentials. In many *Ascaris* preparations, the stimulation of the nerve cord region with carefully placed electrodes gives rise to phasic hyperpolarizations which bring the membrane potential above 40 mV, thus suppressing the generation of rhythmic spike potentials (del Castillo *et al.*, 1963b). These muscle inhibitory potentials, which will be described in detail elsewhere, seem to be due to the stimulation of nerve cord fibres, in agreement with the observations of Goodwin & Vaughan Williams (1963) who first reported the occurrence of muscle inhibition following

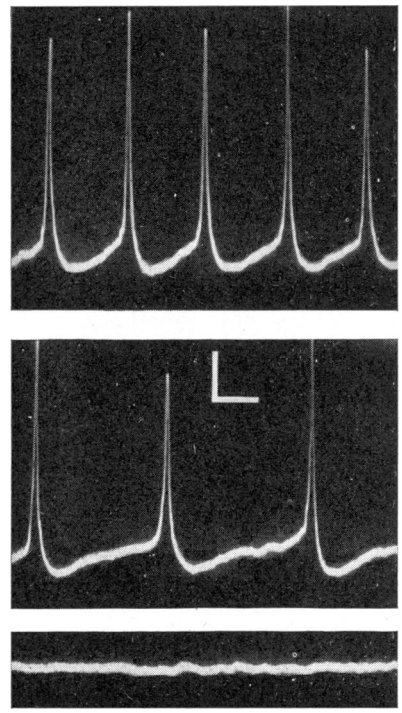

Fig. 4. Suppression of spike activity in an *Ascaris* muscle cell upon addition of piperazine to the bath. Rhythmic spike activity was blocked rapidly without great changes in spike amplitude. Control record (uppermost), taken in 30% sea water; resting potential 27 mV; average spike amplitude just below this value; frequency about 2.9 spikes/sec. Middle record, taken about 1 min after addition of piperazine (5×10^{-4}); the spike frequency had decreased to about 1.6 spikes/sec, while the average amplitude had decreased only slightly. The spikes disappeared suddenly (lowest record) without further decrease in size. Calibrations: vertical, 5 mV; horizontal, 0.2 sec.

the electrical stimulation of the nerve ring situated in the anterior end of *Ascaris*. In our experiments, the inhibitory potentials were often associated with spike potentials believed to be a consequence of the direct electrical excitation of the muscle syncytium.

Experiments in which piperazine was applied while inhibitory potentials were being recorded showed that this compound does not interfere with the function of the inhibitory fibres and synapses. In fact, the suppression of spike activity following the inhibitory stimulus was lengthened in many preparations after the addition of piperazine. This is illustrated by Fig. 6. The upper record was taken with the preparation immersed in 30% sea water. A single electric shock applied to the nerve cord region resulted in a spike (marked by the arrow) followed by a small hyperpolarization. Although a longer than average interval is seen between this spike and the following one, it resembles more the compensatory pause following an extrasystole than genuine inhibition (a longer-lasting hyperpolarization

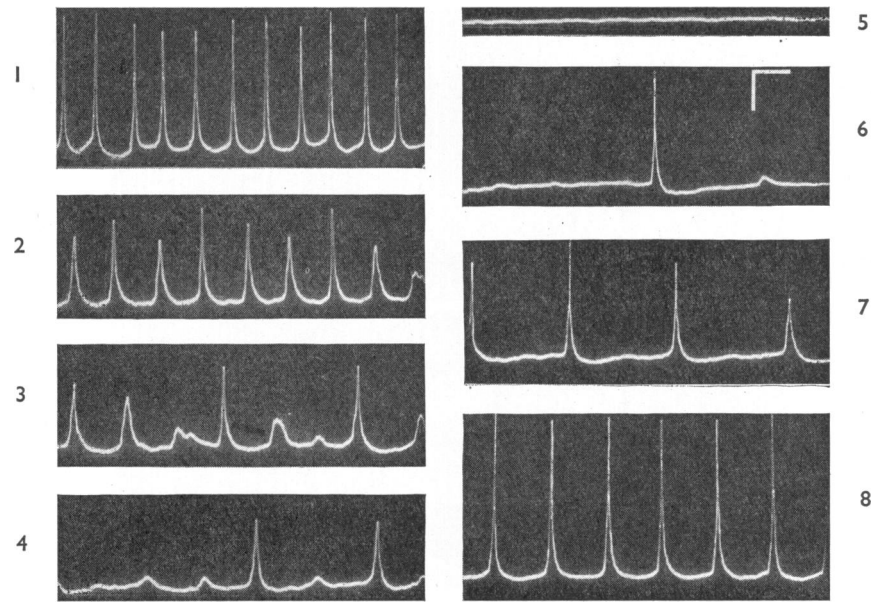

Fig. 5. Suppression by piperazine of the electrical activity of an *Ascaris* muscle cell and recovery upon removal of the drug. (1) control activity in 30% sea water; resting potential about 35 mV; spike frequency 5.7 spikes/sec; average spike amplitude about 35.3 mV. Between (1) and (2) piperazine was added to the bath in a concentration of 6.6×10^{-4}. The amplitude of the spikes was depressed more rapidly than their frequency. In (4) taken just before the spike activity was completely blocked, the average size of the spikes was of about 14% of the initial value, while their frequency was about 70% of that in (1). (5) shows complete absence of spike activity: resting potential 48 mV. Between (5) and (6) piperazine was removed from the bath: the spike amplitude recovered before the spike frequency. In (8) for instance, the average amplitude of the spikes was about 22% greater than in (1), due to the increased resting potential, while their frequency was only of about 57% of the initial value. Calibrations: vertical, 10 mV; horizontal, 0.2 sec.

and suppression of spike activity could be elicited by repetitive stimuli delivered with the electrodes in the same position). The lower record was taken several minutes after adding piperazine (3.3×10^{-4}) to the bathing solution. Although a slight hyperpolarization had already occurred, and the amplitude of the spikes had decreased, their frequency was still unchanged. The stimulus applied to the nerve cord caused a spike similar to that in the upper record, but the ensuing hyperpolarization and inhibition were considerably larger than those observed before the addition of the drug. Furthermore, the frequency of the spikes remained slowed down for a considerable period following their suppression.

The above effect cannot be explained on the basis of an increase in either the amplitude or the duration of the inhibitory potentials. On the contrary, they decrease when the cells become hyperpolarized because of the reduced difference between the membrane potential and the equilibrium level for inhibition.

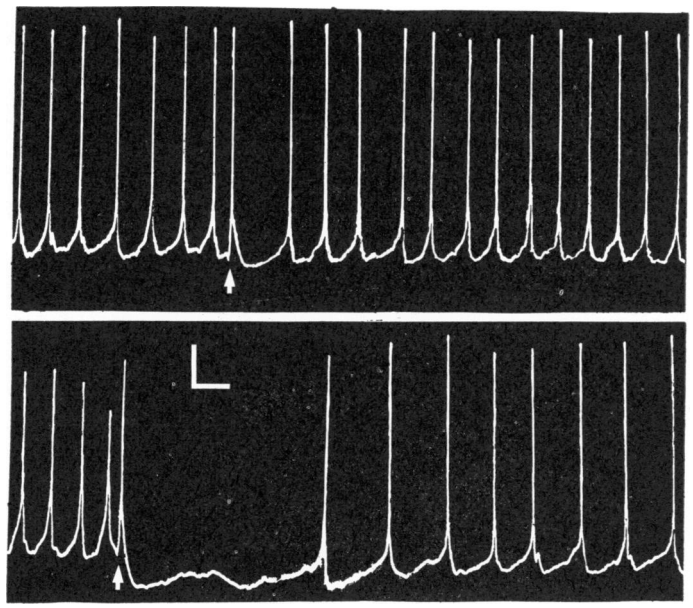

Fig. 6. Influence of piperazine on the inhibitory effects following the electrical stimulation of the nerve cord region in an *Ascaris* preparation. The upper record was obtained with the preparation in 30% sea water. The stimulus gave rise to a spike potential, marked by the arrow, followed by a small hyperpolarization and a longer interval before the next spike was fired. The lower record was taken from the same cell belly after addition of piperazine (3.3×10^{-4}) to the bath. The position of the stimulating electrodes remained unchanged. A period of spike suppression, lasting nearly 3 sec, followed the stimulus and the initial spike (marked by the arrow). The frequency of the rhythmic potentials fell after the period of spike suppression. These changes may be interpreted as an increase in the degree of " postinhibitory polarization " (see text). Calibrations: vertical, 5 mV; horizontal, 0.5 sec.

The record A and its continuation B, in Fig. 7, show also a prolonged suppression of spike activity under the influence of piperazine. The cell was hyperpolarized and the frequency of the spike potentials was reduced. In record B, a postinhibitory facilitation, that is an increase in the spike frequency, can be observed, a rather common occurrence in our preparations. Records C and D were taken from the same cell soon after the spike activity had been completely blocked by the continuing action of piperazine. In contrast to A and B, the electrical stimulation of the nerve cord region with the electrodes in the same position produced only short inhibitory potentials, whose time course cannot account for the long duration of spike suppression in A and B.

We feel inclined to construe the prolonged inhibitory effects observed in the presence of piperazine as a form of *postinhibitory polarization* or *depression,* a term introduced by Kuffler & Eyzaguirre (1955) to describe the delayed hyperpolarization and suppression of spike activity which follow the stimulation of the inhibitory axon in isolated neurone preparations from the stretch receptor of the crayfish. Although

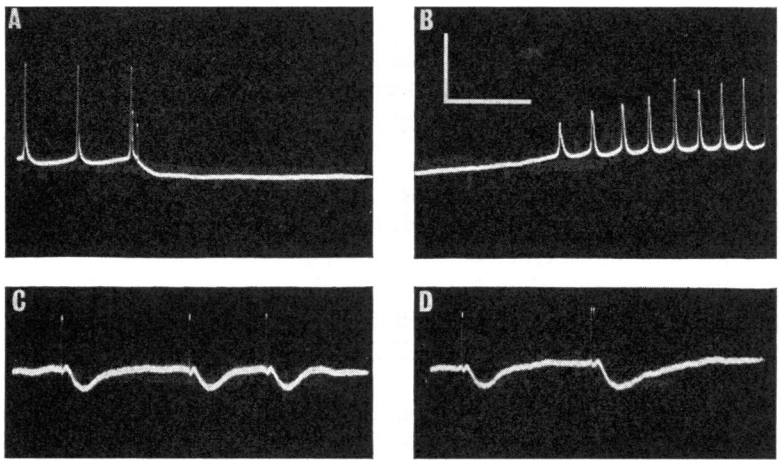

Fig. 7. Effects of electrical stimulation of inhibitory nerve cord fibres in the presence of piperazine (6.6×10^{-4}). In A and B (separated by an interval of just over 1 sec) a relatively long suppression of spike activity was elicited by two stimuli applied to the nerve cord region. The spike suppression was followed by a period of " postinhibitory facilitation " (see text). C and D were taken from the same cell belly (at an amplification twice that used in A and B) after the spike activity had been blocked by the continued action of piperazine. Five inhibitory potentials are seen; the second one in D, which has a slightly longer duration, was elicited by two successive stimuli of the same strength as in A. Calibrations: vertical, 10 mV in A and B, 5 mV in C and D; horizontal, for all records, 0.5 sec.

we have observed this phenomenon in the absence of piperazine, its duration increases markedly under the influence of this drug.

In some of the experiments described by Kuffler & Eyzaguirre (1955) the intervals separating the first few spikes after the postinhibitory polarization are lengthened, that is the rate of rise of the prepotentials is slowed down. This is what is shown in the lower record of Fig. 6. In other neurones, however, they observed an increased frequency of firing after postinhibitory polarization (*postinhibitory facilitation*). As we have mentioned (see Fig. 7, B) this also occurred in many of our preparations.

Nature of the membrane changes induced by piperazine. The above experiments show that the changes induced by piperazine in *Ascaris* muscle cells are similar to those produced by the stimulation of inhibitory nerve fibres. Therefore, the question was raised whether the pharmacological inhibition of *Ascaris* muscle involves changes in membrane permeability similar to those known to be produced in other excitable cells by inhibitory synaptic transmitters. An increase in the resting potential of a cell can be brought about by an enhancement of the permeability of its surface membrane to either potassium or chloride. In either instance, the resulting movement of ions along existing concentration gradients results in an increased negativity of the cytoplasm.

To see whether similar mechanisms were involved in the paralysing action of piperazine, experiments were conducted in which the hyperpolarization produced by a given concentration of this compound was measured with the preparation

immersed in solutions containing different concentrations of potassium and chloride ions. After control tests in the usual 30% sea water, the preparations were placed in the modified solutions and allowed to equilibrate, for at least 15 min, with the new ionic environment, the solution being renewed two or three times during this period.

(*a*) *Changes in the external potassium concentration.* In our experimental conditions, the resting potential of *Ascaris* muscle cells appeared to be determined by the concentration of external chloride ions, as well as by the ratio between intra- and extracellular potassium ion concentrations (de Mello *et al.*, 1963); thus, the potassium content of the external solution could be changed over a relatively wide range without depolarizing the cells. Table 2 shows the average muscle

TABLE 2

HYPERPOLARIZING ACTION OF PIPERAZINE ON *ASCARIS* MUSCLE CELLS IMMERSED IN POTASSIUM-POOR (3 mM) AND POTASSIUM-RICH (40 mM) SOLUTIONS

Each preparation was equilibrated for at least 15 min, with the solutions indicated in columns 1 to 5. The last column gives the piperazine concentration in solutions 2 and 5. Other values are the membrane potential in mV given as means and standard errors for groups of twenty cells (medial bellies) at 39 to 40° C. In experiments 3 and 4, originally performed with a different purpose, no measurements were taken in solutions 3 and 4

	Bath solution					
Expt.	1 Low-K^+	2 Piperazine in low-K^+	3 Low-K^+	4 High-K^+	5 Piperazine in high-K^+	Piperazine concentration
1	33·8±1·51	40·3±1·18	33·4±1·12	34·4±1·67	43·9±1·97	$6{\cdot}6\times10^{-4}$
2	33·2±1·79	39·2±0·92	33·6±1·04	33·9±1·59	39·1±1·13	$6{\cdot}6\times10^{-4}$
3	30·1±1·08	42·3±0·78	—	—	39·9±0·83	$6{\cdot}6\times10^{-4}$
4	39·8±1·13	51·4±1·46	—	—	51·0±1·21	$1{\cdot}3\times10^{-3}$

resting potential of preparations equilibrated with solutions containing 3 and 40 mM-potassium ions and the effect of adding piperazine to both solutions. No significant differences were seen. In other experiments, the effects of piperazine on preparations equilibrated with potassium-free solutions were compared with those observed when the same preparations were immersed in 30% sea water. These experiments also gave negative results.

(*b*) *Changes in the concentration of extracellular chloride ions.* In contrast to the above results, the hyperpolarizing action of piperazine was found to depend on the concentration of chloride ions in the surrounding medium. Solutions with decreasing chloride content were prepared by mixing, in various proportions, 30% artificial sea water containing 175 mM-chloride ions with a 30% solution of an artificial sea water in which all the sodium chloride had been replaced by an isosmotic solution of sodium sulphate. The chloride content of this solution was of 40.4 mM.

The first change observed when piperazine was added to preparations surrounded by a solution with reduced chloride content was a lengthening of the time required for the suppression of rhythmic spike activity. Thus, when piperazine (10^{-3}) was added to a preparation equilibrated with normal 30% sea water, inhibition of rhythmic spike activity occurred usually in 2 or 3 min. However, in preparations exposed to the same concentration of piperazine in a solution containing 107.8 mM-

chloride ions (that is 61.5% of that in 30% sea water) the spike activity persisted for about 15 min.

As shown in Table 3 this slower rate of action of piperazine is associated with a decreased hyperpolarization. The first two columns of this table give the average

TABLE 3

INFLUENCE OF THE EXTRACELLULAR CONCENTRATION OF CHLORIDE IONS ON THE HYPERPOLARIZATION PRODUCED BY PIPERAZINE IN *ASCARIS* MUSCLE CELLS

Membrane potentials in mV are given as means and standard errors of groups of twenty cells at 39 to 40° C. The preparations were equilibrated successively with the numbered bath solutions containing: (1) 175 mM-chloride ions; (2) concentrations of chloride ions given in the last column; (3) piperazine in solution (2); and (4) piperazine in solution (1). The piperazine concentration was $1{\cdot}3\times10^{-3}$ in all experiments. * This value, possibly due to intense spontaneous activity of the inhibitory synapses, is the highest recorded in 30% sea water; the subsequent behaviour of this preparation fully agreed with the other experiments

	Bath solution				Chloride concentration in solutions 2 and 3 (mM)
	1	2	3	4	
Expt.	Normal-Cl^-	Low-Cl^-	Piperazine in low-Cl^-	Piperazine in normal-Cl^-	
1	26·9±1·08	18·0±1·39	23·7±1·61	38·4±1·05	85
2	31·6±0·87	24·4±0·87	32·9±1·21	45·6±1·03	85
3	33·2±0·97	22·5±1·21	24·3±0·91	42·4±0·99	58
4	33·4±1·52	19·5±1·18	21·0±0·84	35·0±1·32	58
5	33·2±1·19	22·3±1·02	28·8±1·01	49·5±1·08	41
6	52·2±1·16*	19·9±0·69	25·8±1·17	43·1±0·97	41

resting potential of *Ascaris* muscle cells in 30% sea water and low-chloride solution respectively. A considerable depolarization occurred when the external chloride concentration was reduced. The third column shows the effect of adding piperazine (10^{-3}) to the low chloride solutions. A hyperpolarization still occurred, but it was insufficient, in most instances, to restore the average muscle membrane potential to the initial value in 30% sea water. The fourth column in Table 3 shows the effect of restoring the chloride concentration to the initial value (175 mM) while keeping the concentration of piperazine constant. This was done by washing the preparation with 30% normal sea water containing piperazine (1.3×10^{-3}). A large hyperpolarization followed within 1 or 2 min.

The conclusion can be drawn that the electric charge necessary to increase the negativity of the cytoplasm upon the addition of piperazine is likely to be carried by an inward movement of chloride ions across the cell surface membrane. In other words, it appears that the immediate effect of piperazine upon *Ascaris* muscle cells is to increase the conductivity of their membrane to chloride.

Although most of the values given in the third column of Table 3 are lower than the corresponding ones in the first column, they still lie within the range of membrane potentials in which rhythmic spike potentials are generated by *Ascaris* muscle (del Castillo *et al.*, 1963a). In spite of this, prolonged exposure of the preparations to piperazine in low-chloride solutions often results in a suppression of the rhythmic spikes which is not associated with further changes in resting potential. It appears, therefore, that an increase in the chloride permeability of the muscle membrane,

while the extracellular concentration of these ions is reduced, interferes with the pace-making mechanisms, which are likely to involve periodic changes in such permeability.

DISCUSSION

The experimental results described above have shown that the paralysing action of piperazine on *Ascaris* muscle cells is due to a substantial rise in the electrical potential difference between their cytoplasm and the external solution. The observations made on preparations bathed in solutions with modified chloride concentrations suggest, furthermore, that such hyperpolarization is brought about by a specific increase in the permeability of the cell surface membrane to chloride ions. The increase in the membrane potential is accompanied by a reduction in both the frequency and ampltude of the rhythmic spike potentials, which disappear when the average value of the former exceeds about 40 mV.

These changes are analogous to those produced in other excitable cells by the action of inhibitory synaptic transmitters, and to the inhibitory effects observed in *Ascaris* muscle cells after electrical stimulation of the nerve cord region. It may be concluded, therefore, that piperazine acts as a pharmacological analogue of a natural inhibitory neurohormone.

This view is also supported by the fact that the membrane changes caused by piperazine appear to take place in the region of the muscle syncytium, where the junctions between inhibitory nerve cord fibres and the muscle membrane are believed to be located. It is not unreasonable to assume that the effects of piperazine may be due to the activation of the same inhibitory postsynaptic receptors which are involved in the neural control of muscle activity.

Although in our experiments the inward membrane current responsible for the hyperpolarization appears to be transported by chloride ions, it is questionable whether this also occurs when ascarids are paralysed inside the human or animal intestine after the therapeutic administration of piperazine. As we have seen, both the resting potential and the hyperpolarizing action of piperazine are reduced when the external chloride ions are replaced by larger, nonpenetrating anions. Yet the perienteric fluid of fresh *Ascaris* specimens not exposed to artificial saline solutions contains only 52 mM-chloride ions (Hobson *et al.*, 1952) that is about one-third of that in 30% sea water. It may be concluded, therefore, that anions other than chloride must be responsible for the transport of the charge necessary to maintain and increase the membrane potential.

Chemical analysis of the ionic content of the perienteric fluid of *Ascaris* has revealed the existence of high concentrations of volatile fatty acids containing from one to seven carbon atoms (see, among others, Bueding, 1953 ; Ellison, Thomson & Strong, 1960). Recent experiments in this laboratory (del Castillo, de Mello & Morales, unpublished) have shown that the resting potential of *Ascaris* muscle is maintained, or even increased, when two-thirds of the extracellular chloride ions are replaced by volatile fatty acids. It is possible, therefore, that the paralysis of *Ascaris* after therapeutic administration of piperazine may be due to the inward movement of organic anions across the cell surface membrane.

Bueding, Saz & Farrow (1959) have reported that incubation of *Ascaris* with paralysing concentrations of piperazine greatly reduces the production of succinic acid, a compound synthesized in large quantities by this organism under both aerobic and anaerobic conditions. In fact, a close parallelism was observed between the concentrations of piperazine necessary to paralyse *Ascaris* muscle, those which block the effects of acetylcholine on this tissue (Norton & DeBeer, 1957) and those which decrease succinate production. Whether or not the decreased synthesis of succinate is a consequence of the inhibition of spontaneous electrical and mechanical activity of *Ascaris* muscle, or a parallel but unrelated phenomenon, is a problem open to both speculation and further research.

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