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THE EFFECT OF DEPOLARIZATION ON THE RATE CONSTANTS OF THE HYOSCINE-ACETYLCHOLINE ANTAGONISM STUDIED ON THE LONGITUDINAL MUSCLE OF THE GUINEA-PIG'S ILEUM

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It is generally admitted that the stimulating effect of acetylcholine and other agonists in the normal, polarized muscle, involves the prior combination of the molecule with a " receptor " on the cell membrane. The same receptor is also the binding site of the specific competitive antagonists. The quantitative study of these inter-relationships has given a much more accurate insight of the drug-cell interaction.

It is now well known that depolarized vertebrate smooth muscle also contracts under the action of acetylcholine (Evans, Schild & Thesleff, 1958) and carbachol (Durbin & Jenkinson, 1961), and the same authors have already shown that atropine blocks these effects. Schild (1964) also mentions that depolarization does not affect the blocking activity of atropine, mepyramine, piperoxane and tubocurarine.

There are a number of papers dealing with these relationships in the normal, polarized smooth muscle, but in the depolarized smooth muscle the only quantitative study we know is that of Schild (1964) on the adrenergic receptor. This author observed that the pA_2 of piperoxane is not changed by depolarization.

The purpose of this paper is to study quantitatively the antagonism between hyoscine and acetylcholine in the depolarized isolated longitudinal muscle of the guinea-pig's ileum. These experiments were done in order to throw some light on the drug-cell interaction under these particular conditions.

Of all the methods described for this study, Paton's (1961) seems to be the more developed, and allows the calculation not only of the equilibrium constant (K_e) but also of the forward (k_1) and backward (k_2) rate constants for the reaction between the competitive antagonists and the cell receptor.

We followed this method, whose theory has already been described (Paton, 1961 ; Paton & Rothschild, 1965 ; Paton & Rang, 1965, 1966), so that we will only use here its final formulae and calculations.

Some preliminary results were presented to the Sociedade Portuguesa de Biologia.

THEORY

By the mass-action law, which the drug-receptor interaction is believed to follow, the rate of the receptor occupation by the antagonist is given by:

$$\frac{dq}{dt} = k_1 x (1 - q) - k_2 q$$

k_1 and k_2 being the forward and backward rate constants, x the concentration of the antagonist and q the proportion of receptors occupied by the antagonist.

Integration of this equation gives:

$$q = q_e \left\{1 - \exp \left[- (k_1 x + k_2) t\right]\right\}$$

where q_e is the proportion of receptors occupied at equilibrium. The onset rate constant $-(k_1 x+k_2)$ can be calculated from the plot of $\log_{10} (q_e-q)$ vs. t which gives a straight line with a slope equal to $-0.4343 (k_1 x+k_2)$.

q was calculated from the relation:

$$q = \frac{DR - 1}{DR}$$

in which DR is the ratio of equi-active concentrations of acetylcholine in presence of the antagonist and when alone.

At equilibrium

$$q_e = \frac{DR_e - 1}{DR_e} \text{ and } K_e = \frac{x}{DR_e - 1}$$

When the antagonist is being washed out, the number of occupied receptors declines following the equation:

$$q = q_e \left\{ \exp \left[-k_2 t\right]\right\}$$

The plot of $\log_{10}$ q vs. t gives, in the same way, a straight line with a slope equal to $-0.4343 k_2$.

Another advantage of this calculation method is the possibility of an internal cross-checking of the results which was done, as Paton (1961) describes, by calculating the ratio between the value of K_e measured in equilibrium and the quotient of $\frac{k_2}{k_1}$. This ratio should be 1.

METHODS

We chose hyoscine as antagonist because Paton (1961) found it to be more specific than atropine for the cholinergic receptor.

The test organ, a strip of the longitudinal muscle of the guinea-pig's ileum (Rang, 1964), was used in order to reduce delays due to diffusion. With the normal, polarized, muscle we did two groups of experiments, one at 37° C, and the other at 22° C. The first one provided the control values for the equilibrium and rate constants, the second one gave the results to be compared with the values obtained in the depolarized muscle. This lower temperature was chosen to avoid the intense contracture of the depolarized preparation which develops in the presence of Ca++ at 37° C (Evans *et al.*, 1958).

The experiments with the polarized muscle were done using normal Krebs with the following composition (mM): NaCl 113; KCl 4.7; $CaCl_2$ 2.54; $MgSO_4$ 2.44; $NaHCO_3$ 24.8; KH_2PO_4 1.2; glucose 11.5. All reagents were analytical grade and were dissolved in borosilicate-glass-distilled water.

The experiments with the depolarized muscle were done in a modified Krebs, in which the NaCl was substituted by an equi-osmotic K_2SO_4 concentration (90 mM) and the $NaHCO_3$ by an equi-osmotic $KHCO_3$ concentration (24.4 mM).

The equi-osmotic concentrations of K_2SO_4 and $KHCO_3$ were calculated from the International Critical Tables (1928). We used acetylcholine and hyoscine bromides from BDH. The acetylcholine solutions were made fresh daily and those of hyoscine twice a week, in double-distilled water.

The strip was tied to a stainless steel rod and mounted in a 10 ml. water-jacketed bath at a constant temperature of 22° C.

Both the Krebs reservoir and the bath were bubbled with 95% O_2+5% CO_2. The pH of both solutions was 7.4 at 22° C.

The muscle contraction was recorded on a smoked drum by means of a very light balsa wood auxotonic frontal lever (Paton, 1957) with a static load of 30 mg and a dynamic load of 80 mg/cm. The lever was mechanically vibrated in order to avoid any stickiness of the point on the drum and to decrease friction as much as possible.

In the Krebs-potassium sulphate experiments, the strip was given at least 45 min to depolarize and for the initial contracture to wear off before the acetylcholine was added to the bath.

The drugs were applied with an automatic syringe in the following way: acetylcholine, which was injected every minute in the polarized muscle and every 2 min in the depolarized preparation, remained in the bath for 20 sec and was then washed out for 4 sec. The wash employed 45 ml. of solution, which was previously seen to be enough to flush out completely the colour of methylene blue. The washing was always done by overflow, in order to avoid cooling or drying the tissue. When hyoscine was being used, it was replaced immediately after each washing-out of acetylcholine.

All concentrations are expressed as molarities and the units of k_1 are M^{-1} sec^{-1}, those of k_2 are sec^{-1} and those of K_e are M.

The experiment began with the determination of a dose-effect curve, usually of four or five points to give a control. A test concentration was chosen and was repeated at least four times to see if the strip was properly stabilized. If there was any change in the sensitivity of the organ, a new dose-effect curve was obtained.

The hyoscine was then added to the bath. The concentrations used, 2.5, 5 and 10×10^{-9} M were chosen because the block was slow enough to be followed. It was also easily surmountable by concentrations of acetylcholine that were not very large so that any side or non-specific effects should not have been important. The dose-ratio values did not exceed 50. The hyoscine was left in contact with the muscle until equilibrium was reached. The depolarized muscles were then washed out for about 20 min to give the recovery curve. The polarized preparations were washed till the contractions were within 10–15% of the control ones for the same concentration.

The contraction heights were measured to the nearest half millimetre. The control dose-effect curve was plotted on semi-logarithmical graph-paper and the ratios of the equi-active concentrations (DR) were calculated. From these we got the proportion (q) of receptors occupied by hyoscine. The onset rate constant (k_{on}) was calculated from the slope of the best straight line fitted to the plot of $\log_{10}(q_e-q)$ vs. t. The equilibrium constant (K_e) was calculated from the equilibrium dose-ratio (DR_e). The dissociation rate constant (k_2) was also obtained from the slope of the straight line fitted to the plot of $\log_{10} q$ vs. t.

RESULTS

Polarized muscle

This series of experiments was done in order to provide the normal values of the constants, as determined at 37° C, and as a basis for comparison with the results obtained with the depolarized muscle at 22° C. At this low temperature the contraction was slower and the muscle slightly less sensitive to acetylcholine. In some preparations the muscle showed a variable amount of spontaneous activity, usually not strong enough

to upset the experiment. However, in some cases this spontaneous activity was accompanied by an increase in the muscular tone and the experiment was abandoned.

In these experimental conditions we observed that the onset and the offset of the antagonism was indeed an exponential process as the theory predicted and as Paton (1961) had already observed. The rate constants found with the normal, polarized, smooth muscle at 37° C and 22° C are shown in Table 1. The ratio between the K_e measured in equilibrium and a value calculated from $\frac{k_2}{k_1}$ is very reasonably close to unity, as expected, being 0.88 at 37° C and 0.83 at 22° C.

TABLE 1

RATE CONSTANTS FOR OCCUPATION OF ACETYLCHOLINE RECEPTORS BY HYOSCINE IN THE POLARIZED AND DEPOLARIZED LONGITUDINAL MUSCLE OF THE GUINEA-PIG'S ILEUM

The results are given as mean±S.E. with the number of observations in brackets.

	Normal polarized muscle		Depolarized muscle	
	37° C	22° C	22° C	
k_1	$1{\cdot}40\pm0{\cdot}21\times10^{6}$ (9)	$0{\cdot}66\pm0{\cdot}07\times10^{6}$ (9)	$0{\cdot}41\pm0{\cdot}05\times10^{6}$ (11)	$M^{-1}\ sec^{-1}$
k_2	$9{\cdot}71\pm1{\cdot}40\times10^{-4}$ (9)	$3{\cdot}10\pm0{\cdot}42\times10^{-4}$ (9)	$0{\cdot}93\pm0{\cdot}01\times10^{-4}$ (11)	sec^{-1}
K_e	$0{\cdot}61\pm0{\cdot}08\times10^{-9}$ (9)	$0{\cdot}41\pm0{\cdot}07\times10^{-9}$ (9)	$0{\cdot}25\pm0{\cdot}03\times10^{-9}$ (11)	M
$\frac{K_e}{k_2/k_1}$	0·88	0·83	1·07	

Depolarized muscle

When the strip of the longitudinal muscle of the guinea-pig's ileum is immersed in K_2SO_4-Krebs it first contracts quickly and then relaxes. We allowed 45 min for the preparation to equilibrate before any drug was added to the bath. The depolarized muscle was slightly less sensitive to acetylcholine and contracted and relaxed more slowly than the polarized one. For this reason the acetylcholine was applied every 2 min. In some preliminary experiments we observed that the depolarized muscle gave steady contractions on application of acetylcholine during at least 3 hr, with variations smaller than 15% of the initial responses.

In order to see if the antagonism between acetylcholine and hyoscine in the depolarized muscle was still competitive, we drew the plot of $(DR_e - 1)$ against the respective concentrations of hyoscine. We made 27 determinations with hyoscine: 8 with 2.5×10^{-9} M; 9 with 5×10^{-9} M; and 10 with 10×10^{-9} M. The means of these determinations are shown in Fig. 1. The straight line calculated by the least squares method is shown as a continuous line and has a slope of 4.61×10^{9}. The theoretical line that passes through the origin is shown as an interrupted line and has a slope of 4.55×10^{9}. The slopes of the two lines are not significantly different ($P>0.9$). The value of the equilibrium constant calculated from this slope is equal to 0.22×10^{-9} M.

In Fig. 2 we show the occupation of the receptors by hyoscine as a function of time. The results are given as mean±s.e. for each time. Each point is the mean of 5 experiments with 5×10^{-9} M and 6 with 10×10^{-9} M hyoscine. This figure is given to show that the experimental points fall on straight lines and that the slopes are dependent on

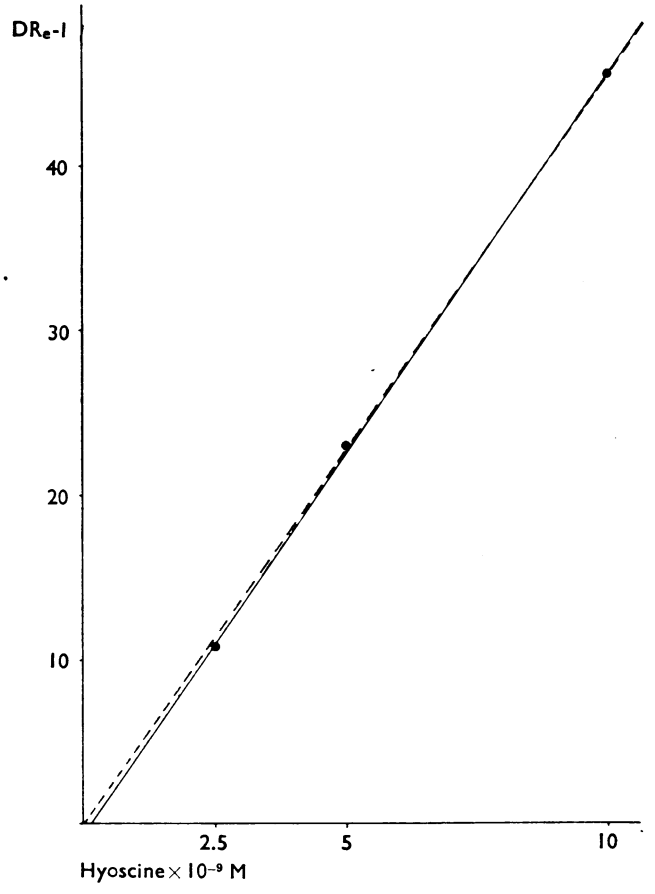

Fig. 1. Plot of the equation $(DR_e-1)=\frac{1}{K_e}$ [hyoscine]. Continuous line = best fit line by the least squares method; interrupted line = theoretical line passing through the origin. Each point is the mean of 8–10 experiments.

the concentration of the antagonist. The higher the concentration the steeper the slope. These facts agree with the theory and show that the process is similar to that observed in the normal muscle.

No calculations were done on this graph. The actual values of the rate constants were obtained from each individual experiment. Figure 3 represents the offset of the antagonism as receptor occupation dwindles after washing out the hyoscine. The points show the means of the results obtained in the experiments. The standard errors are not drawn because the limits are very small (about 1% of the mean).

The rate constants in the depolarized muscle are shown in Table 1. They were calculated in the same way as for the polarized one. The ratio of the value of K_e, as measured after equilibrium has been reached, to the quotient of $\frac{k_2}{k_1}$ is also closer to unity (1.07) than that found with the polarized preparation.

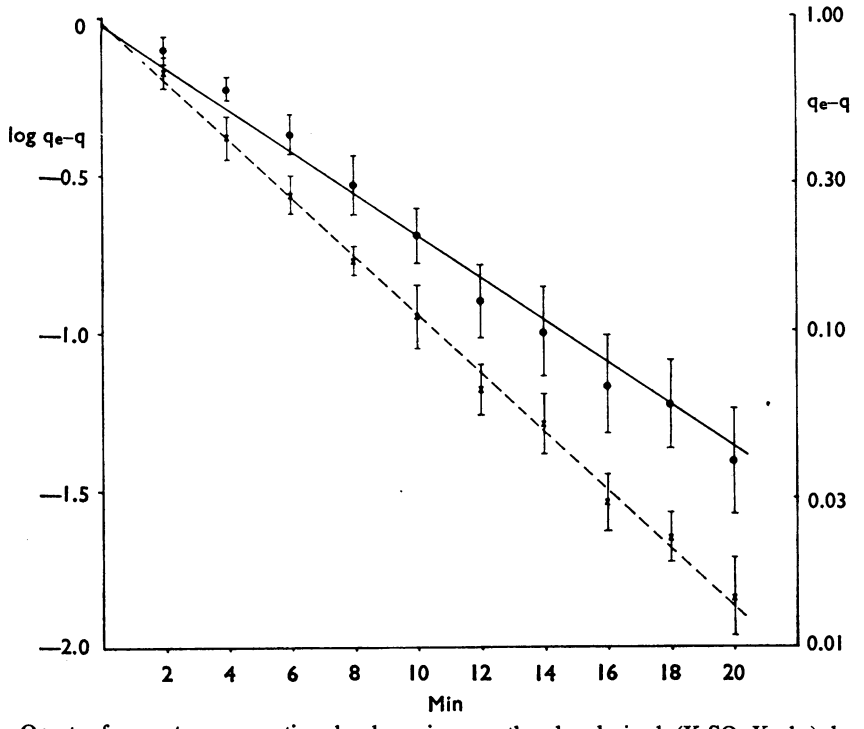

Fig. 2. Onset of receptor occupation by hyoscine on the depolarized (K_2SO_4-Krebs) longitudinal muscle of the guinea-pig's ileum at 22° C. The logarithm of q_e-q is plotted against time (see Theory). Continuous line$=5\times10^{-9}$ M hyoscine ; interrupted line$=10\times10^{-9}$ M hyoscine.

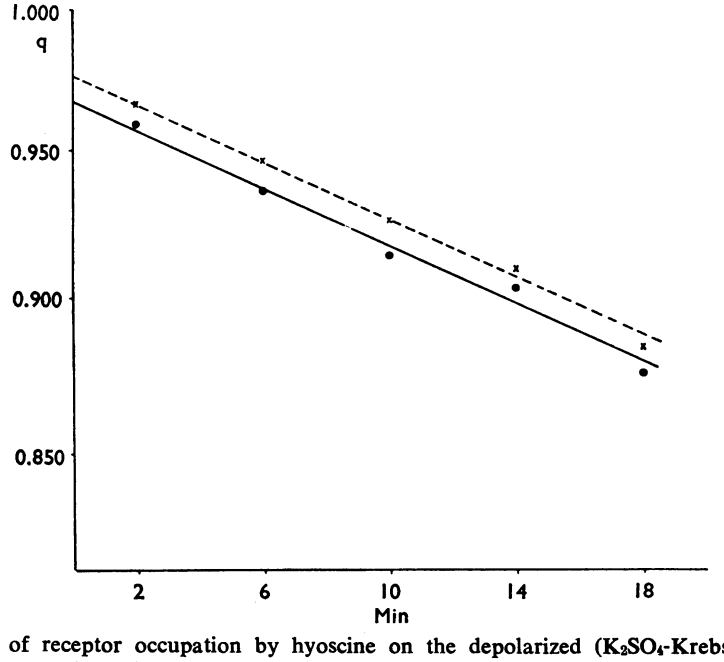

Fig. 3. Offset of receptor occupation by hyoscine on the depolarized (K_2SO_4-Krebs) longitudinal muscle of the guinea-pig's ileum at 22° C. The proportion of receptors occupied has been plotted on a logarithmic scale against time. ●=mean of 5 experiments with 5×10^{-9} M hyoscine ; ×=mean of 6 experiments with 10×10^{-9} M hyoscine.

DISCUSSION

The onset and offset of the antagonism are exponential processes as Paton had already observed. Thus the rates seem to depend only on the velocity of the formation and dissociation of the complex between hyoscine and the receptor.

On the cholinergic receptor Evans *et al.* (1958) had already seen that atropine blocked the acetylcholine-induced contraction of the depolarized chick amnion. Durbin & Jenkinson (1961) have made a similar observation with the depolarized taenia coli stimulated by carbachol. We obtained an analogous result with the more specific hyoscine on the depolarized longitudinal muscle of the guinea-pig's ileum.

Figure 1 shows the plot of the experimental equilibrium dose-ratios minus one against the hyoscine concentrations in the depolarized muscle. The experimental slope does not significantly differ from the theoretical one ($P>0.9$). This suggests that in the depolarized muscle the antagonism between hyoscine and acetylcholine is still present and it is still competitive. The difference between the equilibrium constant in the polarized muscle ($K_e = 0.41 \pm 0.07 \times 10^{-9}$ M) and in the depolarized one ($K_e = 0.25 \pm 0.03 \times 10^{-9}$ M) shows only that depolarization renders the muscle more sensitive to the hyoscine but does not give any indication on the rates of the reaction. Paton's method, allowing the calculation of the rate constants of the reaction, permits a more detailed knowledge of the process. Table 1 shows that in the depolarized preparation the association rate constant (k_1) was slightly but significantly lower than the k_1 of the polarized muscle ($P<0.01$). The k_2 values also fell ($P<0.01$), the dissociation rate constant in the depolarized muscle being nearly three times smaller than in the polarized one. Consequently the equilibrium constant (K_e) is also smaller in the depolarized muscle. The greater stability of the hyoscine-receptor complex in the depolarized muscle may be related to the loss of membrane potential, to a metabolic alteration, or to the different ionic strength of the K_2SO_4-Krebs. The high concentration of $SO_4^=$ ions may have reduced the level of ionized calcium and this could also conceivably affect the kinetics of the action of hyoscine. Thus, Paton & Rothschild (1965) observed that a low Ca^{++} lowers both rate constants but did not change the K_e value. In our case, however, not only the rate constants but also K_e were smaller in the depolarized muscle.

SUMMARY

1. The rate constants of the reaction of hyoscine with the acetylcholine receptor of the polarized and depolarized longitudinal muscle of the guinea-pig's ileum were studied at 22° C.

2. In the depolarized preparation the antagonism between hyoscine and acetylcholine was competitive.

3. In the depolarized muscle the association rate constant (k_1) and particularly the dissociation rate constant (k_2) were smaller than in the polarized muscle. The depolarized preparation was nearly twice as sensitive to the blocking action of hyoscine.

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REFERENCES

DURBIN, R. P. & JENKINSON, D. H. (1961). The effect of carbachol on the permeability of depolarized smooth muscle to inorganic ions. *J. Physiol., Lond.*, **157**, 74–89.

EVANS, D. H. L., SCHILD, H. O. & THESLEFF, S. (1958). Effects of drugs on depolarized plain muscle *J. Physiol., Lond.*, **143**, 474–485.

INTERNATIONAL CRITICAL TABLES OF NUMERICAL DATA (PHYSICS, CHEMISTRY AND TECHNOLOGY) (1928). Vol. IV—National Research Council U.S.A. McGraw-Hill, New York.

PATON, W. D. M. (1957). A pendulum auxotonic lever. *J. Physiol., Lond.*, **137**, 35–36P.

PATON, W. D. M. (1961). A theory of drug action based on the rate of drug receptor combination. *Proc. R. Soc. B.*, **154**, 21–69.

PATON, W. D. M. & ROTHSCHILD, A. M. (1965). The effect of varying calcium concentration on the kinetic constants of hyoscine and mepyramine antagonism. *Br. J. Pharmac. Chemother.*, **24**, 432–436.

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.

PATON, W. D. M. & RANG, H. P. (1966). A kinetic approach to the mechanism of drug action. *Adv. Drug Res.*, **3**, 57–80.

RANG, H. P. (1964). Stimulant actions of volatile anaesthetics on smooth muscle. *Br. J. Pharmac. Chemother.*, **22**, 356–365.

SCHILD, H. O. (1964). Calcium and the effects of drugs on depolarized smooth muscle. *Proc. 2nd Int. Pharmac. Meeting*, **6**, 95–104.