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THE CALCIUM ANTAGONISTIC EFFECTS OF CYPROHEPTADINE ON CONTRACTION, MEMBRANE ELECTRICAL EVENTS AND CALCIUM INFLUX IN THE GUINEA-PIG TAENIA COLI

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1 The ability of cyproheptadine (Cph) to inhibit membrane translocation of calcium in smooth muscle was investigated by studying the drug's action on contraction, electrical activity and calcium influx in the guinea-pig taenia coli.

2 Cph $\geq 10^{-6}$M reduced the amplitude of normal spontaneous contractions and concurrently decreased the number of action potentials occurring with each slow-wave of depolarization (sucrose-gap recordings). These inhibitory effects of Cph were antagonized by increasing the medium [Ca] three fold to 7.68 mM.

3 Intracellular recordings showed that Cph $\geq 2 \times 10^{-6}$M decreased the amplitude and extended the duration of the action potential. These effects were only partially reversible in normal medium whereas large overshooting action potentials were again seen in 7.68 mM Ca medium.

4 High frequency mechanical activity was produced by inclusion of veratridine 5×10^{-6}M in the perfusate. Low concentrations of Cph ($\geq 10^{-7}$M) reduced the amplitude of such contractions at a faster rate than they did normal spontaneous contractions.

5 At concentrations between 10^{-7} and 10^{-6}M, Cph fully reduced the tonic component of contractions elicited in 112 mM isotonic KCl whilst having little or no effect on either (i) the initial phasic KCl contraction or (ii) the 'repolarization contracture' normally produced on wash-out of the KCl or (iii) the spontaneous contractions before and after KCl treatment. In contrast, at Cph 2×10^{-6}M, the repolarization contracture, as well as the isotonic KCl contraction, was totally blocked whereas spontaneous contractions were still unaffected. Progressively higher Cph concentrations inhibited all components of this contractile cycle.

6 Dose-response curves for the rate of drug-induced relaxation of tonic contractures produced in hypertonic 42.7 mM high-potassium medium, showed the calcium antagonistic potency of Cph to be intermediate between that of chlorpromazine and D600. The minimum Cph concentration for effect lay between 1 and 5×10^{-7}M, and the effects of Cph 2×10^{-6}M (approximately the ID_{50}) were totally antagonized by 12.8 mM Ca.

7 By means of a lanthanum wash procedure, Cph $\geq 2 \times 10^{-6}$M was found to decrease the ^{45}Ca uptake occurring into strips of taenia coli in normal medium, although the maximum effect (at Cph 10^{-5}M) amounted to only 25% inhibition of the uptake occurring into control strips (also found with D600). The increased uptake occurring in hypertonic 44.7 mM high-potassium medium was inhibited in a dose-dependent manner by Cph 1×10^{-7}M.

8 The results are consistent with an action of Cph in reducing the flow of Ca^{2+} through voltage-dependent Ca channels in the smooth muscle cell membrane. It is suggested that the interaction of Cph molecules with such sites is dependent upon membrane potential as well as drug concentration.

Introduction

Cyproheptadine (Cph) is already well characterized as a potent 5-hydroxytryptamine and histamine antagonist (Stone, Wenger, Ludden, Stavorski & Ross, 1961). In this paper we describe experiments on the guinea-pig taenia coli which indicate that Cph also blocks Ca movements through excitable membranes, apparently by an action on voltage-dependent Ca channels. Such experiments were suggested by the finding that Cph inhibits both insulin release and calcium uptake resulting from stimulation of pan-

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creatic islets with certain secretagogues thought to act via voltage-sensitive Ca channels in the islet β-cell membrane (high glucose and K^+), whilst leaving the action of other agents not relying on gated entry of Ca unaffected (Richardson, 1976; Joost, Beckmann, Holze, Lenzen, Pozer & Hasselblatt, 1976; Donatsch, Lowe, Richardson & Taylor, 1980). The smooth muscle cells of the guinea-pig taenia coli seem well suited for demonstrating drug action on the membrane translocation of Ca, since they exhibit Ca-dependent action potentials (Tomita, 1975) that are blocked by well characterized Ca antagonists such as verapamil (Golenhofen & Lammel, 1972; Riemer, Dörfler, Mayer & Ulbrecht, 1974) and Mn^{2+} (Nonomura, Hotta & Ohashi, 1966). Cph was found to reduce the magnitude of Ca-dependent mechanical contraction, to inhibit overall spike activity in the tissue (sucrose gap recording), to reduce the amplitude and extend the duration of the intracellularly recorded action potential, and to inhibit ^{45}Ca uptake as measured by a lanthanum wash procedure. These observations establish the ability of Cph to interfere with transmembrane Ca movements through voltage-sensitive Ca channels.

Methods

Male and female guinea-pigs of 300–600 g body weight were used.

Solutions

Krebs Ringer bicarbonate (KRB) was used for experiments in which mechanical and electrical activity was studied, whereas Tris buffered solutions were employed for Ca uptake experiments. The composition of the various solutions is given in Table 1. Twice distilled water was used throughout for solution preparation. Media of increased [Ca] were prepared by addition of solid $CaCl_2$ to the appropriate solution; Na_2HPO_4 was omitted where necessary to avoid problems of precipitation.

Isometric tension recordings

Guinea-pigs were stunned by a blow on the head and strips of taenia coli 1.5–2 cm in length isolated from the caecum. Strips were suspended vertically between a fixed point within the organ bath and a Statham tension transducer by means of cotton threads. Solutions were pumped through the bath continuously at constant rate by means of a peristaltic pump. To expose muscles to different solutions either the pump feeder tube was transferred between reservoirs or drug was added to a single reservoir. Organ baths of 3 or 0.5 ml capacity were employed, with flow rates of 6 and 1 ml/min respectively. The muscle strips were placed under a resting tension of 200–500 mg and left for at least 1 h before beginning experiments. All solutions were bubbled vigorously with 95% O_2 and 5% CO_2. Isometric tension was recorded on a chart recorder. Temperature was controlled at 37°C.

Recording of electrical activity

Sucrose-gap technique Measurements of changes in membrane potential were made with a sucrose-gap chamber which employed pressed rubber membranes for compartment isolation (Boev & Golenhofen, 1974). Changes in muscle tension were recorded by fixing one end of the muscle and attaching the other end by thread to a force-displacement transducer. Membrane potential changes were recorded through silver-silver chloride electrodes connected to the input of a differential amplifier. Both electrical and mechanical responses of the tissue were recorded simultaneously on a two-channel pen recorder. The muscle cells could be exposed to different solutions either by changing from one reservoir to another or by direct addition of drugs to a single reservoir. All experiments were carried out with a continuous flow of Krebs solution (solution 1, Table 1) at room temperature (22°C) or 37°C.

Microelectrode technique Intracellular recordings of membrane potential and electrical activity in the

Table 1 Composition of solutions (mM)

Solution	*NaCl*	*$NaHCO_3$*	*KCl*	*$MgCl_2$*	*$CaCl_2$*	*Na_2HPO_4*	*NaH_2PO_4*	*D-Glucose*	*Tris*	*pH*
1 Normal KRB (1)	116	25	4.7	1.13	2.56	1.42	—	11	—	7.5
2 Normal KRB (2)	137	11.9	2.7	1.00	2.56	—	0.4	5.5	—	7.2
3 Isotonic KCl	—	25	120.7	1.13	2.56	1.42	—	11	—	7.5
4 Hypertonic KCl	137	11.9	42.7	1.00	2.56	—	0.4	5.5	—	7.2
5 Normal Tris HCl	141	—	4.7	1.13	2.56	—	—	11	10	7.4
6 KCl Tris HCl	141	—	44.7	1.13	2.56	—	—	11	10	7.4

KRB = Krebs Ringer bicarbonate solution.

smooth muscle cells of taenia coli were obtained by methods previously described (Bülbring, 1954; Matthews & Sutter, 1967). Glass microelectrodes filled with K-citrate 1.5 M and having a resistance >50 MΩ were used for cellular impalement. Signals were amplified, displayed on an oscilloscope and photographed. All experiments were carried out in Krebs solution (solution 1; Table 1) at 37°C.

^{45}Ca uptake experiments

A modification of the lanthanum wash procedure (van Breemen, Farinas, Gerba & McNaughton, 1972) was employed for these measurements. Such a method is necessary with smooth muscle because changes in intracellular Ca^{2+} pools can only be detected after displacement of the large membrane bound Ca^{2+} compartment with La^{3+} (Brading & Widdicombe, 1977). The radioactive solutions were prepared by adding 3–5 μCi/ml of $^{45}CaCl_2$ (sp. act. 22 mCi/mg; Radiochemical Centre, Amersham) to either normal Tris buffer (solution 5, Table 1) for resting Ca uptake, or KCl Tris buffer (solution 6, Table 1) for K-stimulated Ca uptake; both solutions were saturated with 100% O_2 before addition of the isotope. To a portion of this radioactive solution, a small volume of stock Cph (10^{-2}M) in distilled water was added to bring the final concentration in the buffer to 10^{-4}M. Portions of this solution were then diluted with the remaining buffer solution containing ^{45}Ca to give lower Cph concentrations.

Isolated strips of taenia coli 1–1.5 cm in length were mounted with thin cotton threads on glass rods and pre-incubated for 30 min in batches of two or three in 5 ml of normal Tris buffer (solution 5, Table 1) at 37°C under constant bubbling with 100% O_2. The muscles were then incubated for 1 h in 5 ml of either normal or KCl Tris buffer (solutions 5 or 6, Table 1) containing ^{45}Ca and Cph; incubation was again at 37°C and with bubbling of 100% O_2. At the end of the incubation period the preparations were transferred to Ca-free normal Tris buffer containing 10 mM $LaCl_3$ at room temperature. Every 5 min the preparations were replaced in fresh La containing solution. After 50 min of La-wash the muscle strips were cut free from their cotton ties, blotted and weighed by difference in glass scintillation vials (Beckmann). The tissue was dissolved directly in the vial in 0.5 ml of Soluene-100 tissue solubilizing fluid (Packard Instrument Co., Illinois, U.S.A.) at 60°C for 1 h. Glacial acetic acid, 20 μl, was added to prevent chemiluminescence before adding 10 ml of scintillation fluid (Insta-gel, Packard, Zürich, Switzerland). The ^{45}Ca activity of the tissue was determined by liquid scintillation spectrometry and subsequently the counting efficiency of each vial determined by the addition of an internal ^{45}Ca standard. The resulting correction factors were very close to 1.0 in each case. Total tissue counts per mg wet weight were corrected for isotope dilution (assessed by counting 20 μl samples of each incubation medium) and the net tissue Ca retention expressed as pmol per mg wet weight and per hour (pmol mg^{-1} wet wt. h^{-1}). Student's *t* test was used to examine the statistical significance of mean differences between groups.

Drugs

Cph (Merck), D600 (Knoll), methysergide (Sandoz) and chlorpromazine (Smith, Kline and French) were dissolved in distilled water at a concentration of 5×10^{-3}M and aliquots of these stock solutions added to KRB appropriately. Experiments with nifedipine (Bayer) were conducted in a dark room with sodium vapour lighting to avoid photolytic destruction of the drug. Veratridine and atropine (both Sandoz) were dissolved either directly in KRB or in 10 μl 1 N HCl to which 5 ml KRB was then added before dilution to the required concentration.

Results

Effect of cyproheptadine on spontaneous contractions

Normal, undriven preparations Spontaneous contractions, 5–13 g in amplitude and occurring at 1–5 min intervals, were produced in normal KRB (solution 1, Table 1) under the conditions employed. The amplitude of spontaneous contractions was reduced by Cph, the minimum concentration for effect being about 2×10^{-6}M. At this concentration contraction amplitude was reduced by only 10–20%, even when preparations were exposed to the drug for 90 min. Most preparations exposed to 2×10^{-6}M Cph achieved a constant contraction amplitude, the time taken for this varying between 25 and 80 min. The rate of block during the inhibitory phase was thus slow and varied somewhat between preparations. Cph 5×10^{-6}M produced in all cases a clear increase in the rate of inhibition (Figure 1) and complete block was usually attained within 40–90 min. The rate of block was dose-dependent (Table 2).

Cph 2 and 5×10^{-6}M usually had no effect on contraction frequency; however, in occasional preparations an increase in frequency occurred (Figure 1) with a concomitant regularization of inter-contraction interval. Cph 10^{-5}M produced a decrease of contraction frequency in most preparations; in others no change occurred.

The effects of Cph were partially reversible when preparations were returned to normal medium before full block had been attained (Figure 1). In contrast, no reversibility occurred in preparations

Table 2 Rate of inhibition of spontaneous contraction amplitude by cyproheptadine (Cph)

	Cph $\times 10^{-6}$ *M*	n	*Mean rate of inhibition* (%/min ± s.e.)*
(i) Undriven preparations	2	8	0.33 ± 0.096
	5	8	1.26 ± 0.21
	10	8	2.13 ± 0.36
	100	2	8.30
(ii) Veratridine (5 μM)-stimulated activity	2	4	1.35 ± 0.16
	10**	6	1.88 ± 0.33
	50**	8	4.43 ± 0.70

*Rate of inhibition was calculated as the reduction in amplitude per minute expressed as a % of the mean amplitude of the eight spontaneous contractions occurring before exposure to Cph. Where possible, rate of inhibition was calculated using the time taken to attain full block. In other cases (especially at 2×10^{-6} M Cph) the inhibition occurring over the total period of inhibition was measured.

**Only those preparations showing a progressive decrease in contraction amplitude (see text) were included in this analysis.

that had been exposed to Cph for sufficiently long to establish full block (no contractions for 10 min).

Exposure of fully blocked preparations (10^{-5}M Cph) to normal medium containing Ca 7.68 mM in the absence of Cph resulted in one of two effects. In the first type of response, the muscle strips began contracting rhythmically again, the amplitude increasing progressively to a level slightly less than pre-Cph values (Figure 2). Alternatively, the response consisted of small rapid contractions superimposed on a tonic contracture. Following such a response to raising the [Ca], return to normal medium restored spontaneous activity. These results demonstrate that raising the medium [Ca] three fold can reactivate the spontaneous contractions blocked by Cph. No such reversibility occurred when the medium [Ca] was increased three times in the continued presence of Cph.

Preparations driven with veratridine Inclusion in the perfusate of veratridine 5×10^{-6}M, a plant alkaloid known to increase the Na permeability of excitable membranes, was found to produce a marked increase in contraction frequency that was sustained over long periods. In addition, contraction amplitude became consistent and was usually increased.

Preparations driven with veratridine were more sensitive to low concentrations of Cph than undriven preparations. The threshold concentration for effect was 10^{-7}M but only 10–20% inhibition of contraction amplitude was produced at this dose. Cph 10^{-6}M was more effective but still did not produce total block. Cph 2×10^{-6}M caused a much greater rate of inhibition than in undriven preparations (Table 2) and full block could be achieved. However, at Cph 10^{-5}M the rate of inhibition of veratridine driven preparations was about the same, or in some cases

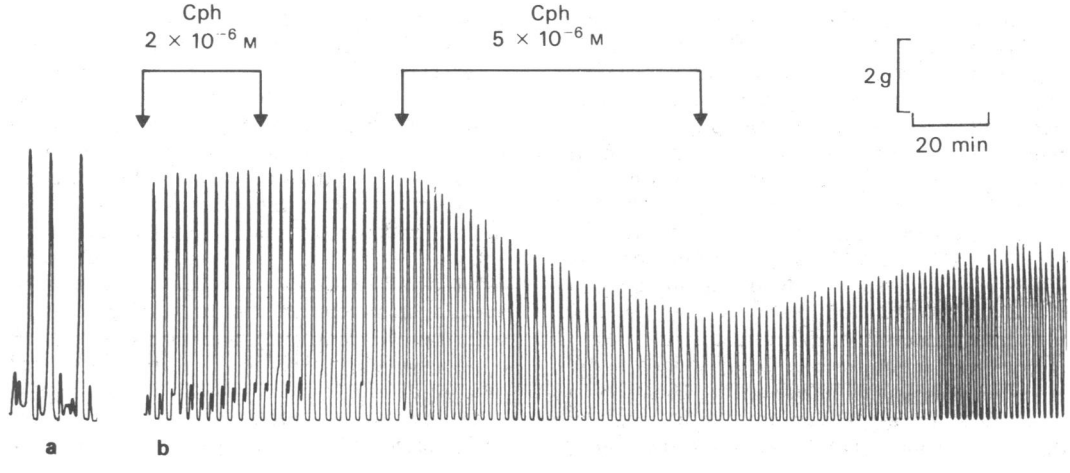

Figure 1 Effect of cyproheptadine (Cph) on spontaneous contraction of guinea-pig taenia coli: (a) typical spontaneous activity recorded before addition of drug; (b) record beginning 60 min after starting perfusion with Cph 2×10^{-6}M.

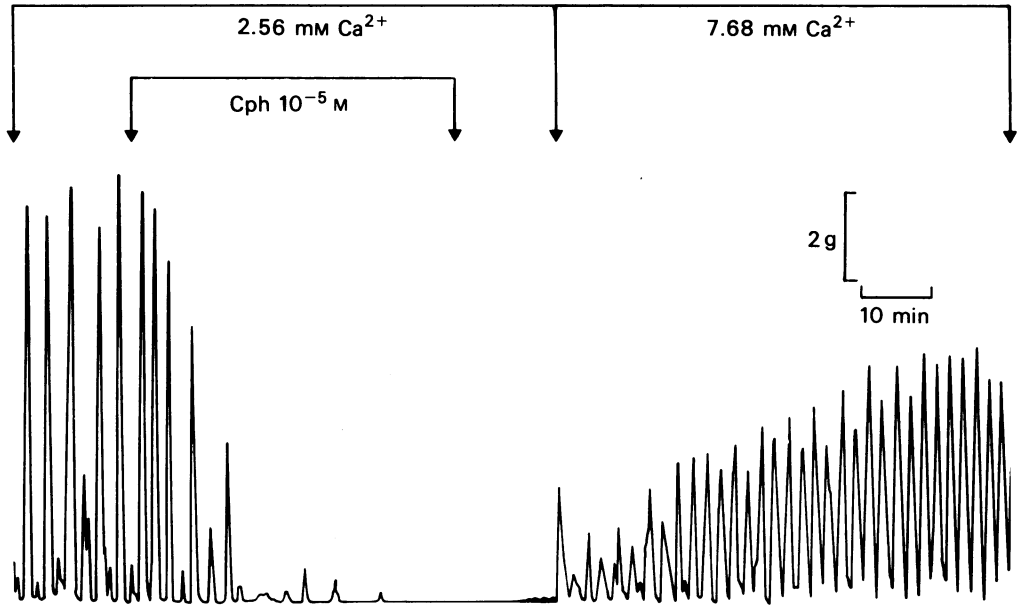

Figure 2 Effect of increasing $[Ca]_o$ on cyproheptadine (Cph)-induced block of spontaneous mechanical activity.

even less than that in non-stimulated preparations. In fact the time course of inhibition of driven preparations exposed to Cph 10^{-5}M showed various forms. In 7 out of 17 experiments, contraction amplitude simply decreased at constant rate to base-line, the rate of inhibition being approximately the same as in undriven preparations (Table 2). Figure 3 illustrates one of two further preparations in which an initial period of rapid inhibition was followed by a final period in which the inhibition was continuous but of relatively low rate. In the remaining experiments, the amplitude decreased progressively until it was one third to one tenth that before Cph treatment, this amplitude being either (1) more or less maintained for 1–2 h before decreasing suddenly to zero, or (2) continuing until the experiments were terminated after 4 h exposure to Cph. A similar behaviour was observed on exposure of some strips to D600 10^{-5}M.

The contraction frequency of veratridine-driven preparations was usually unaffected by Cph, except in a single experiment where Cph 2×10^{-6}M caused a sudden and substantial reduction of this parameter. The effects of Cph were partially (50–90%) reversible even in those preparations exposed to 10^{-5}M or

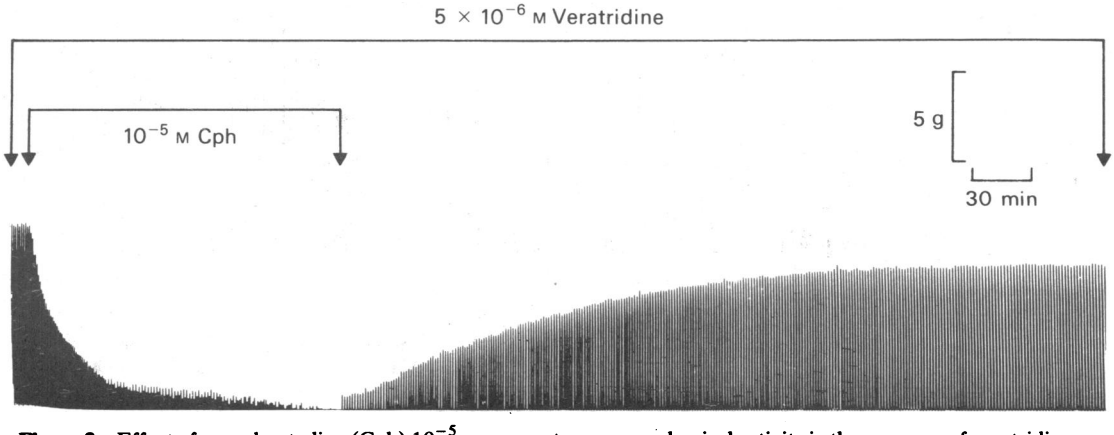

Figure 3 Effect of cyproheptadine (Cph) 10^{-5} M on spontaneous mechanical activity in the presence of veratridine 5×10^{-6} M: note that the amplitude of contractions alternates towards end of inhibitory phase. Reversibility can be better studied in such preparations since possible effects of Cph on pacemaker centres are overcome by the continual driving influence of veratridine.

5×10^{-5}M Cph for sufficiently long to establish full block (Figure 3). Preparations varied, in the time taken for contractions to begin again following removal of Cph and in some cases 30 min elapsed before small amplitude contractions were observed. Similar results were obtained with D600 10^{-6}M. In contrast, atropine 10^{-5}M and methysergide 10^{-6}M, either alone or together, had essentially no effect on contraction amplitude and frequency.

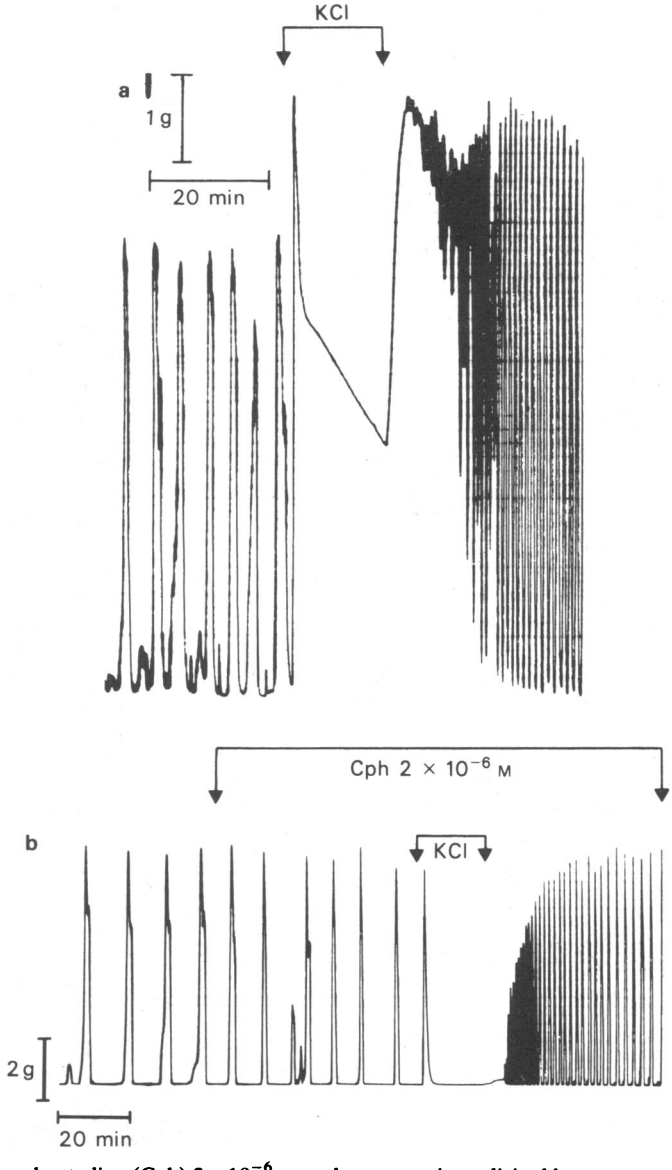

Figure 4 Effect of cyproheptadine (Cph) 2×10^{-6}M on the contractions elicited by exposure to and wash-out of 116 mM isotonic KCl medium. (a) Control. Isotonic KCl produces an initial phasic contraction of the muscle which then relaxes to a tonically maintained level. Reperfusion of normal medium produces a further increase in tension followed by a marked increase in spontaneous contractility. (b) Same contractile cycle as (a) but in the presence of Cph 2×10^{-6}M. The preparation was exposed to drug for 1 h before stimulation with KCl. The tonic tension normally developed in KCl and the repolarization contracture are abolished by pretreatment with Cph 2×10^{-6}M, whereas spontaneous contractions both before and after KCl treatment are unaffected. Different preparation from (a).

Effect of cyproheptadine on contractions evoked by exposure to and wash-out of isotonic KCl medium

Exposure of taenia coli to isotonic KCl (solution 3, Table 1) evokes an initial phasic contraction that rapidly adapts to a lower tonically maintained level (Riemer *et al.*, 1974). As shown in Figure 4a, wash-out of the KCl solution evoked a further substantial increase in tension (repolarization contracture) followed by a marked increase in spontaneous activity (see also Gabella, 1978). The repolarization contracture (RC) is associated with a sustained high frequency discharge of action potentials during which the membrane potential is repolarizing (Holman, 1958). The discharge frequency achieved at the peak of this effect is equal to or even exceeds that produced during the initial exposure to isotonic KCl (Holman, 1958).

The effects of Cph 2×10^{-6}M on this contractile cycle is shown in Figure 4b. As described above, this concentration caused only a small degree of inhibition of the normal spontaneous contractions after exposure to the drug for 1 h. In marked contrast the tonic component of the isotonic KCl contraction as well as the repolarization contracture were completely blocked by Cph 2×10^{-6}M. However, 10–15 min after returning to normal solution (the normal duration of the RC) high frequency spontaneous contractions began that attained the same amplitude as those prior to isotonic KCl exposure. This type of experiment demonstrates that Cph 2×10^{-6}M has a marked differential effect, blocking fully those contractions associated with massive action potential discharge at reduced membrane potentials (RC) whilst leaving spontaneous contractions at normal resting potentials relatively unaffected in amplitude.

Analogous effects were produced by Cph 5×10^{-6}M, although in the initial 60 min of drug exposure, spontaneous activity was reduced more than with Cph 2×10^{-6}M. Cph 10^{-5}M produced block of spontaneous contractions before KCl treatment, as well as the tonic KCl and repolarization contractions, whereas the phasic KCl contraction and a transient burst of small amplitude spontaneous activity following KCl treatment were still present. Lower Cph concentrations produced further interesting effects. Cph 10^{-6}M reduced the tonic component of the isotonic KCl contraction whereas a substantial repolarization contraction was still present; however the repolarization contraction produced in normal medium following the second exposure to KCl was slightly reduced. Cph 2 and 5×10^{-7}M also produced a reduction of the tonic component of the isotonic KCl contraction without noticeable effect on the reactivation contraction, whereas no effect on either of these contractions was observed at Cph 10^{-7}M.

Thus the threshold concentration for an effect of Cph on the tonic KCl contraction, the most sensitive of all components of this contractile cycle, is 2×10^{-7}M. Repolarization contractures are blocked at Cph $> 1-2 \times 10^{-6}$M, spontaneous contractions at Cph $> 5-10 \times 10^{-6}$M, whereas the initial phasic contraction in KCl is still partially present at 10^{-5}M.

Effects of Cph on the various components of the contractile cycle were partially reversible, except in the case of the tonic KCl contracture. Even in preparations that were washed in normal medium for up to 3 h after exposure to Cph 10^{-5}M no tonic tension was produced on exposure to isotonic KCl. The wash periods employed were sufficient to produce substantial recovery of the spontaneous activity and repolarization and phasic KCl contractions.

Effects of cyproheptadine on hypertonic KCl contractures

When exposed to a hypertonic high K^+ solution (42.7 mM), a tonically maintained contracture is developed that is associated with a measurable increase in Ca^{2+} influx (Karaki, Ganeshanandan, Ikeda & Urakawa, 1969; van Breemen, Aaronson & Lontzenhiser, 1979). This offers a suitable basis for examining the concentration/time-dependent effects of smooth muscle relaxants on a more quantitative basis. In these experiments, preparations were left for 1 h in solution 2. They were then contracted in hypertonic high KCl (solution 4) and after 20 min, a given dose of drug was applied in the continued presence of KCl. This produced relaxation that was eventually complete. The rate of inhibition, measured between the times of drug addition and 50%

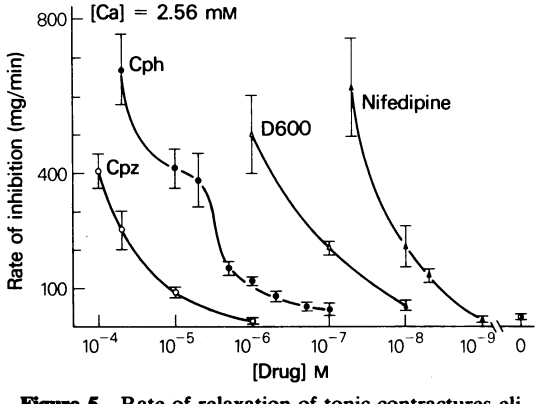

Figure 5 Rate of relaxation of tonic contractures elicited by 42.7 mM hypertonic high K^+ medium as a function of concentration of chlorpromazine (Cpz), cyproheptadine (Cph), D600 and nifedipine. Each point is the mean of four measurements; vertical lines show s.e.mean. Experiments were so arranged that all four determinations for each concentration of a given drug were from different individuals.

inhibition, as a function of concentration is shown in Figure 5 for Cph, D600, nifedipine and chlorpromazine. The threshold concentration for an effect of Cph on this contracture was 2×10^{-7}M. The potency of Cph in antagonizing Ca-dependent contractures in hypertonic high K^+ medium was intermediate between that of D600 and chlorpromazine.

The rate of relaxation produced by Cph 2×10^{-6}M was decreased by raising the medium [Ca] (Figure 6); in these experiments, high calcium was present from the beginning of the experiment and the drug added after depolarization in KCl. The effects of Cph 2×10^{-6}M were totally antagonized by Ca 12.80 mM (i.e. 5 × normal [Ca]).

Interestingly, both the Cph dose-response curve at 2.56 mM as well as the Ca dose-response curve at Cph 2×10^{-6}M appeared biphasic (Figures 5 and 6).

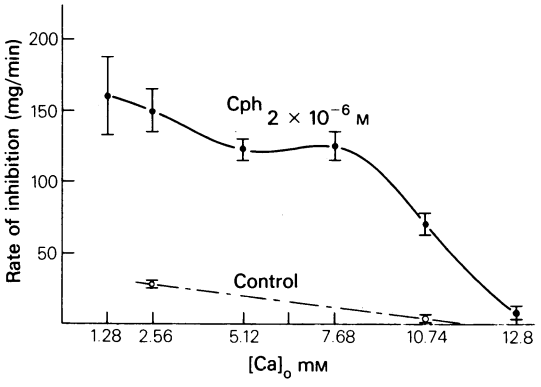

Figure 6 Relaxation rate produced by cyproheptadine (Cph) 2×10^{-6}M of contractures induced in hypertonic 42.7 mM KCl, as a function of medium [Ca]. Each point is the mean of four separate determinations; vertical lines show s.e.mean.

Effects of cyproheptadine on membrane electrical properties

Sucrose-gap studies Recordings of spontaneous activity before addition of drug could be characterized into one of two groups. In one type of preparation (group 1), spontaneous contractions were accompanied by regular bursts of spikes superimposed upon slower changes in membrane potential, depolarization and the onset of spike discharge immediately preceding each period of mechanical contraction. Addition of Cph 2×10^{-6}M caused a progressive decrease in the number of spikes associated with each slow wave of depolarization and a corresponding decrease in the spike frequency within each burst; these inhibitory effects of Cph were usually complete after 30 min exposure to the drug. A second group of preparations, possibly those more sensitive to stretch, exhibited a relatively constant discharge of spikes and a corresponding tonically maintained level of tension; brief periods of relaxation were associated with a decrease in spike frequency (Figure 7a). Exposure of such preparations to Cph 2×10^{-6}M resulted initially in the emergence of rhythmic spontaneous activity (Figure 7b), similar to that recorded from group 1 preparations before Cph treatment. Subsequently the drug reduced the number and frequency of spikes associated with each slow wave of depolarization, in a similar way to its effects on preparations of group 1 (Figure 7c). These effects of Cph were only slowly and partially reversible on washout (Figure 7d). Further exposure to the drug again reversibly blocked electrical and mechanical activity but the inhibitory effects developed much more rapidly (i.e. within 2–3 min following drug exposure) (Figures 7e and f). Increasing the medium [Ca] three fold again antagonized the inhibitory actions of Cph (Figure 7g).

Intracellular studies Spontaneous action potentials of 30 to 50 mV amplitude were recorded intracellularly in all experiments before addition of Cph. In one of two experiments there was a small decrease in amplitude and extension in duration of individual spikes following continuous exposure to Cph 10^{-6}M; the other experiment showed little or no effect even after 60 min. In contrast, Cph 5×10^{-6}M or 10^{-5}M caused such an abrupt arrest of electrical activity, that changes in the spike characteristics were almost impossible to document. Consequently, the effects of Cph 2×10^{-6}M were studied in seven further experiments. In each case there was a decrease in both spike amplitude and spike frequency; individual spikes were also extended in duration. These effects were progressive in any one cell, and after 20–25 min spiking cells were no longer encountered. The blocking effects of Cph were long-lasting and not fully reversed by prolonged washing in normal Krebs solution. Restoration of spike amplitude, frequency and duration was, however, markedly enhanced by a three fold increase in the medium [Ca] i.e. to 7.68 mM. These various effects are illustrated in Figure 8.

Effects of cyproheptadine on Ca uptake

As shown in Figure 9, the mean Ca uptake occurring in normal medium and in 44.7 mM hypertonic high K^+ medium was reduced in a dose-dependent manner by Cph. At 1 mM Ca the difference between control and drug-treated groups was statistically significant ($P<0.05$) at Cph concentrations $>$ 2×10^{-6}M for both normal and KCl-treated groups. In 2.56 mM Ca medium, Cph 2×10^{-6}M also produced a significant reduction of Ca uptake occurring

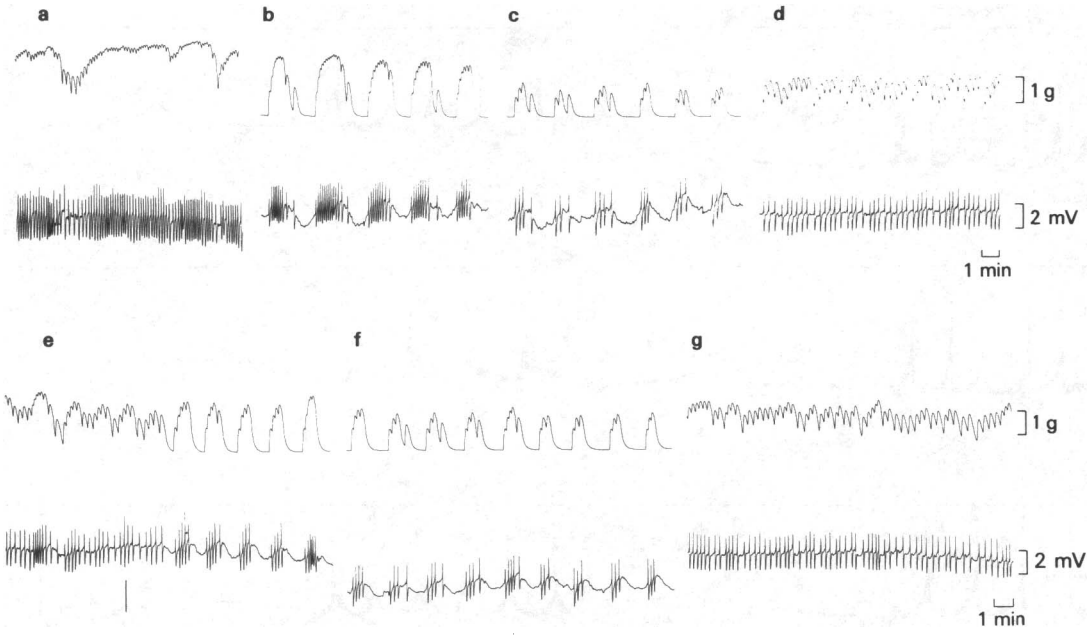

Figure 7 Effects of cyproheptadine (Cph) on the mechanical and electrical activity of taenia coli. Sucrose-gap recordings at 22°C, all from the same experiment. The upper record of each panel is of muscle activity (contraction upwards) and the lower record of electrical activity (action potentials upwards). (a) Before, (b) 50–60 min after, (c) 80–90 min after exposure to Cph 2×10^{-6} M, and (d) 80–80 min after washout of Cph. Record (e) starts 120 min after removal of Cph 2×10^{-6} M which is re-introduced at the arrow. Note the rapid onset of inhibition. Record (f) was obtained 10–30 min after exposure to Cph. The calcium concentration was increased three fold from 2.56 mM to 7.68 mM during and 30 min before (g); Cph was present throughout (f) and (g). Note the reversal of Cph inhibition in (g) by the increase in $[Ca]_o$.

in normal medium but the threshold concentration for an effect on the KCl-induced increment in Ca uptake lay between 1 and 5×10^{-7} M. In both normal media the maximum inhibition of Ca uptake produced by Cph was only 20–25%, whereas full inhibition by Cph of the increment in Ca uptake produced by high K^+ could be observed.

In unstimulated preparations pre-incubated with Cph in normal medium for 2.5 h, inhibition of the KCl-induced increment in Ca uptake also occurred at Cph $> 10^{-7}$ M. Thus a prolonged period of preincubation did not dramatically reduce the threshold concentration for an effect of Cph on Ca uptake.

Discussion

Our results confirm in another tissue the previous suggestion that Cph possesses calcium antagonistic properties (Richardson, 1976). Both spontaneous contractions and contractures elicited by depolarization in high K media were blocked by Cph and these effects could be antagonized by increasing the medium [Ca]. The experiments on veratridine driven preparations show that inhibitory effects of Cph on spontaneous contraction amplitude can occur without changes in contraction frequency, so that effects of Cph on pacemaker centres, although possibly present in some non-driven preparations do not alone underlie the reduction in spontaneous mechanical activity. Spontaneous contractions of the rat uterus are also blocked *in vitro* by Cph at concentrations similar to those employed in our study (Sadovsky, Dora, Pfeifer, Polishuk, Rachamimoff & Sulmam, 1973) showing that these effects are not confined to intestinal smooth muscle. The sucrose gap results demonstrate that such inhibitory effects on mechanical activity are accompanied by a corresponding reduction in electrical spike activity in the tissue, suggesting that uncoupling of membrane electrical from muscle contractile events is unlikely to be involved in the action of Cph. The intracellular recordings show that Cph reduces the amplitude and increases the duration of the Ca-dependent action potential and that increasing the medium [Ca] can overcome these effects. This demonstrates directly that Cph interferes with the flow of Ca^{2+} through the voltage-sensitive channel of the action potential. Similar ef-

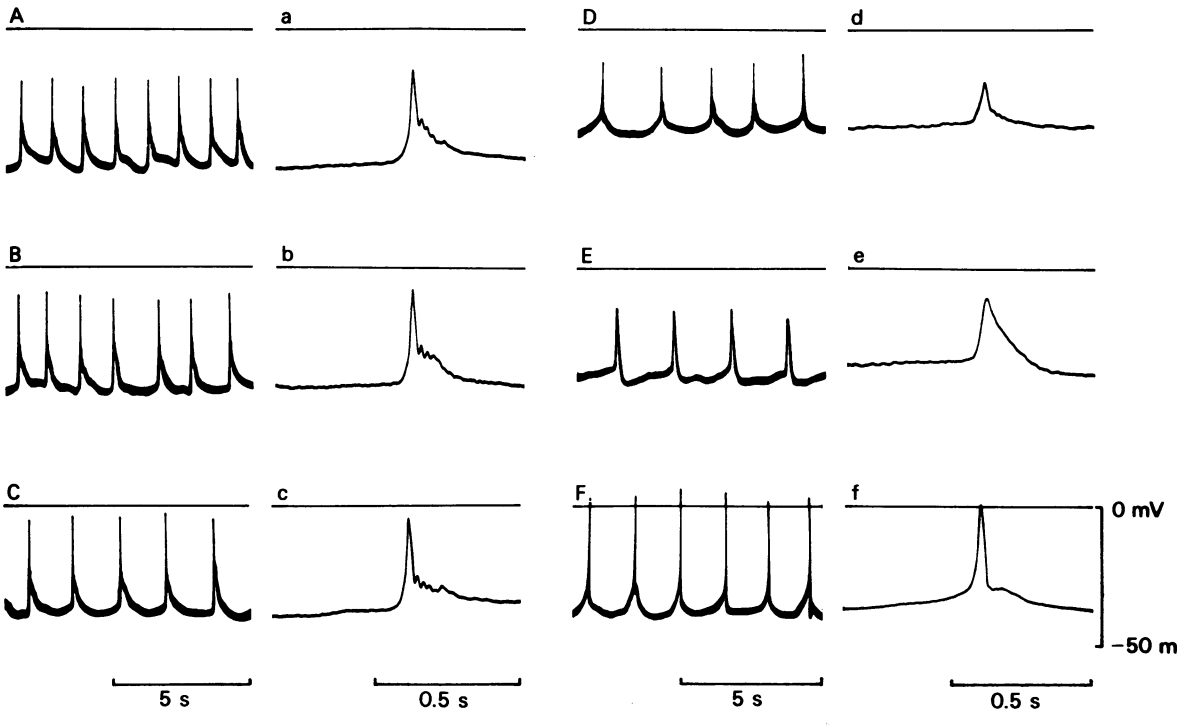

Figure 8 Effect of cyproheptadine (Cph) on electrical activity of taenia coli. Oscilloscope recording of intracellular potentials. (A) Spontaneous activity before, and in (B), (C) and (D) activity 5, 10 and 15 min after exposure to Cph 2×10^{-6} M. (E) shows the activity recorded 70 min after restoration of normal Krebs solution and (F) the subsequent response 22 min after increasing $[Ca^{2+}]_o$ to 7.68 mM. Recordings (A) to (D) are from the same, and (E) and (F), from different cells in the same experiment. Note the decrease in spike amplitude and frequency together with an extension of spike duration following CPH; these effects are reversed by the increase in $[Ca^{2+}]_o$. (a–f) recorded at faster oscilloscope sweep time a few seconds after the corresponding slower record (A–F).

fects were observed on taenia coli with the calcium antagonist, verapamil (Golenhofen & Lammel, 1972; Riemer *et al.*, 1974).

In addition, Cph partially reduced (20–25%) the Ca^{2+} uptake occurring in normal medium. This portion of inhibitable uptake presumably reflects the Ca^{2+} influx that accompanies spontaneous electrical activity, whereas the remainder is due to other processes such as Ca-Ca, Ca-Na exchange and passive Ca permeabilities. In a similar way incubation of taenia for 4 h with D600 2×10^{-5} M produced only 25% inhibition of resting Ca^{2+} uptake (Mayer, van Breemen & Casteels, 1972). More strikingly, the increment in Ca uptake produced by depolarization in 44.7 mM high K^+ medium was blocked in a dose-dependent manner by Cph $> 10^{-7}$ M and full inhibition could be observed. The increase in Ca^{2+} influx induced by hypertonic KCl is thought to occur through the same voltage-sensitive mechanism involved in the action potential (Bolton, 1979). Thus this result is also consistent with the conclusion that Cph blocks voltage-sensitive Ca channels in smooth muscle membranes.

Since Cph is a tertiary amine that will partition into the membrane lipid, a general increase in membrane fluidity resulting in non-specific disturbances of the allosteric lipid-protein arrangements at the Ca channel site might be considered responsible for such Ca antagonistic effects. However, Cph appears 10 to 100 times more active in blocking Ca^{2+} movements than chlorpromazine, a classic tertiary amine membrane stabilizer, despite the fact that the *n*-butanol/water partition coefficient and the acid dissociation constant are approximately the same for the two drugs (our own unpublished observations). In addition the membrane stabilization properties of Cph, as evaluated by nerve conduction blockade experiments begin only at Cph concentrations of 5×10^{-5} M (Riccioppo Neto, 1979; our own unpublished experiments), whereas the calcium antagonistic properties occur between 10^{-7} and 10^{-5} M. Likewise in rat pancreatic islets, high concentrations of Cph have

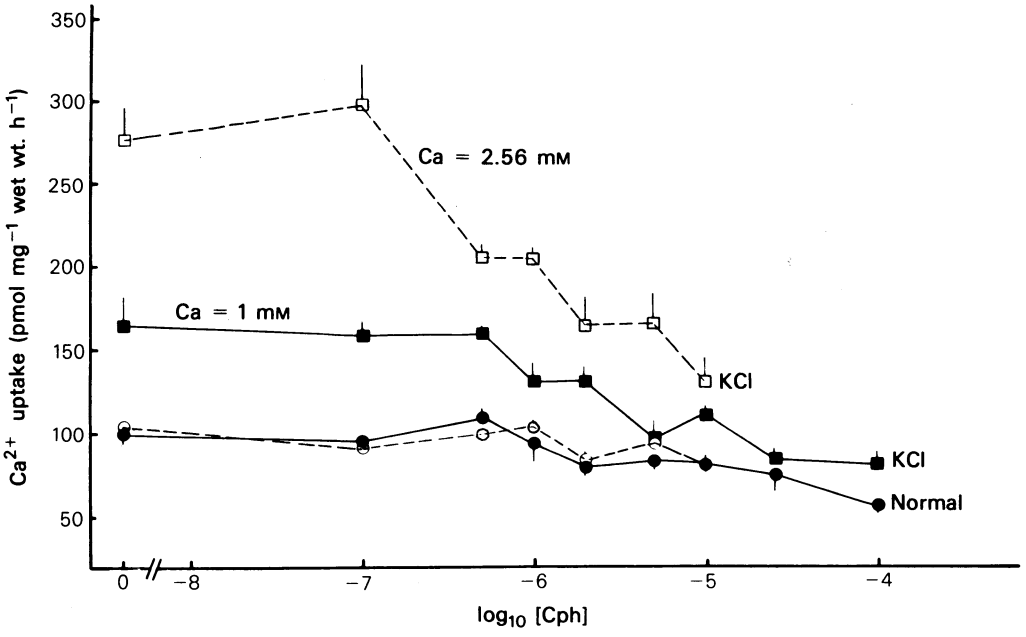

Figure 9 Effect of cyproheptadine (Cph) on calcium uptake measured with a lanthanum wash procedure. Filled symbols = 1 mM Ca medium. Open symbols = 2.56 mM Ca medium. Circles represent Ca uptake in normal medium and squares that occurring in 44.7 mM hypertonic high K^+ medium. Each point represents the mean and vertical line represents s.e.mean. For each point in the 1 mM Ca experiment, $n = 8$. In the experiment with 2.56 mM media, $n = 12$, except for the control preparations exposed only to normal medium where $n = 20$.

only a small effect on $^{22}Na^+$ influx through tetrodotoxin-sensitive Na channels opened by veratridine, whereas total inhibition of Ca^{2+} influx through Ca channels opened by glucose and high K^+ is observed (Donatsch *et al.*, 1980). Thus the weight of evidence favours the view that non-specific membrane stabilization effects are not predominantly involved in the Ca antagonistic properties of Cph and that we are dealing with a fairly, but not totally, selective calcium antagonistic agent.

Several details of our results suggest that the interaction between Ca channels and Cph molecules is use and membrane potential-dependent. Firstly in the spontaneous contraction experiments, increasing the contraction frequency with veratridine resulted in a ten fold reduction of the minimum Cph concentration for effect and at low concentrations increased the rate of contraction amplitude inhibition. This is rather similar to the action of verapamil on heart muscle in which the degree of inhibition produced by a given concentration increases, the greater the contraction frequency (McCans, Lindenmayer, Munsen, Evans & Schwartz, 1974; Bayer, Hennekes, Kaufmann & Mannhold, 1975). Of course veratridine probably causes some degree of membrane depolarization so this result does not allow formal separation of use from depolarization-dependent inhibition by Cph. However, it is very interesting to note that the inhibitory effects of a given concentration of Cph on nerve compound action potentials are also increased when the preparation is driven at higher stimulation rates (Riccioppo Neto, 1979). Thus Cph appears to interfere with cation movement through both Ca and Na channels in a way whose effectiveness is dependent on the frequency of opening (and/or closing) of such channels. The mechanism of this remains to be elucidated but the simplest hypothesis is that the drug interacts more effectively with the open rather than the closed channel.

In addition to use-dependent inhibition, two lines of evidence point to a membrane potential-dependent interaction between Ca channels and Cph. In the first place the threshold concentration for an effect of Cph on tonic contractures and Ca uptake induced by 44.7 mM hypertonic high K^+ medium was 10 to 20 times less than that required for an effect on normal contractions. It is well known that high $[K^+]$ depolarizes the smooth muscle membrane and the resulting Ca influx is thought to occur via the same route as is is normally activated during the upstroke of the action potential (Bolton, 1979).

The second piece of evidence comes from the contraction experiments with isotonic KCl. Here Cph 2×10^{-6} M fully blocked both the KCl tonic contrac-

ture and the repolarization contracture normally occurring on wash-out of KCl, whilst leaving the spontaneous activity before and after KCl treatment more or less unaffected (Figure 4b). The repolarization contracture is associated with massive action potential discharge at reduced membrane potentials (Holman, 1958) and is blocked by Cph 2×10^{-6} M, whereas the spontaneous contractions, which are also associated with action potential discharge but occur at normal membrane potentials, remain unaffected by the drug. In addition, the increase in contraction amplitude of Cph-treated preparations during wash-out of the KCl has a similar time-course to that for re-establishment of the membrane potential (Holman, 1958; Katase & Tomita, 1972). Thus the ability of Cph 2×10^{-6} M to block selectively the repolarization contracture appears related to the reduced membrane potential pertaining during that period. This suggests that the drug has an increased affinity for Ca channels at reduced membrane potentials. Differential effects of this kind can be observed at Cph 2×10^{-6} M because this concentration is sufficiently low to allow separation of membrane potential and concentration-dependent binding.

It should be added that such differential effects on the tonic KCl and repolarization contracture are also seen with D600 and verapamil but not nifedipine (our own unpublished observations). Thus the mechanism of action of Cph is likely to be similar to that of D600. Indeed a recent voltage clamp study by McDonald, Pelzer & Trautwein (1980) has demonstrated that blockade of heart Ca channels by D600 increases when the channel is activated and that removal of block is much faster in the rested state than when the channel is depolarized. This mechanism of action is similar to that of local anaesthetics on Na channels (Hille, 1977; Hondeghem & Katzung, 1977). Thus compounds like D600 and Cph may differ from local anaesthetics only in their greater selectivity for Ca as compared to Na channels.

In summary, we conclude that Cph blocks the voltage-sensitive Ca channels of smooth muscle that are normally activated during the early phase of the action potential. The affinity of the drug for such channels appears dependent upon their frequency of activation as well as membrane potential. This mode of action is similar to D600 and verapamil. Since changes in Ca channel kinetics arise in some systems as a consequence of activation of 5-hydroxytryptamine receptors (Klein & Kandel, 1978; Pellmar & Carpenter, 1979) it is tempting to speculate that the anti-5-hydroxytryptamine and anti-histamine properties of Cph arise partially due to its ability to block Ca channels, especially those held in the open configuration by receptor activation.

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