Br. J. Pharmac. (1969), 36, 549–560.

Blockade or reversal of the contraction induced by calcium and adrenaline in depolarized arterial smooth muscle

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1. Mesenteric arteries immersed in a depolarizing solution contract in the presence of calcium. These contractions are proportional to the calcium concentration and are reversible.

2. Mesenteric arteries immersed in a calcium-free depolarizing solution contract in the presence of adrenaline. Under the experimental conditions reported here, this response develops only about one-third of the contractile tension developed in polarizing solution (modified Krebs bicarbonate).

3. Cinnarizine and chlorpromazine inhibit the contractile response to calcium and induce relaxation of depolarized muscle previously contracted by calcium ; cinnarizine was 4 times more potent than chlorpromazine in such activity.

4. Chlorpromazine inhibits the response to adrenaline in both polarizing and calcium-free depolarizing solutions, whereas cinnarizine inhibits the response in polarizing solution but not that in calcium-free depolarizing solution.

5. The significance of these results is discussed.

The contractile response of vascular smooth muscle to depolarization by potassium is reduced by phenoxybenzamine, dibenamine and chlorpromazine (Bevan, Osher & Su, 1963 ; Shibata & Carrier, 1967 ; Godfraind & Polster, 1968), desipramine (Hrdina & Garattini, 1967), cinnarizine (Godfraind, Kaba & Polster, 1968) and lidoflazine (Godfraind & Polster, 1968). The inhibitory effect is dependent upon the calcium concentration in the perfusion fluid.

Cinnarizine (1-benzhydryl-4-cinnamylpiperazine, dihydrochloride) has vasodilating properties (see, for example, Godfraind *et al.*, 1968) and is an antagonist of adrenaline, angiotensin and 5-hydroxytryptamine. Chlorpromazine also has potent anti-adrenergic activity (Ariens & Simonis, 1964) and it inhibits the action of the sarcoplasmic calcium pump (Bazler & Makinose, 1968). Cinnarizine and chlorpromazine were used as antagonists in the present experiments in order to determine whether their inhibitory properties are due to antagonism of the function of calcium in the contractile process of vascular smooth muscle.

Arterial smooth muscle preparations were depolarized in the absence of free calcium ions and the contraction evoked by subsequent addition of calcium or adrenaline was studied by adding known amounts of these compounds to the bath fluid. The ability of cinnarizine and chlorpromazine to prevent such muscular contraction, or to abolish it once established, was investigated. The effects of the

two antagonists on adrenaline-induced contraction of polarized muscle were also studied.

A preliminary communication on some of this work was made at the Chelsea meeting of the British Pharmacological Society in January 1969.

Methods

Isolated vascular preparations

All experiments were performed on rabbit mesenteric arteries which are highly sensitive to the action of the drugs to be used (Godfraind *et al.*, 1968). The animals (weight about 1·5 kg) were killed by a blow on the head and exsanguination. Care was taken in the selection of arteries for assay as the sensitivity of such preparations to antagonists varies with the size of the vessels (Godfraind *et al.*, 1968). The arterial muscle studied comprised sections of the major branches of the mesenteric artery (outside diameter 1 mm), the posterior duodenal artery (0·8–0·5 mm), and collaterals of the ileocoli-caecal trunk (0·7–0·4 mm).

Vessel strips 4 cm long were prepared by spiral section, suspended in a 50 ml. organ bath containing modified Krebs bicarbonate at 37° C, and gassed with a mixture of oxygen (95%) and carbon dioxide (5%). Assays were performed 2 hr after dissection.

Recordings

Recordings of the responses of the 1 mm arteries were made by isometric lever using two strain gauges as part of a balanced bridge, the output of which was fed into a potentiometric recorder.

Recordings of the responses of the other preparations were made using a displacement transducer (Hewlet Packard 7 DCDT-1000), the output of which was also fed into a potentiometric recorder. (The transducer was built to record isotonic contractions ; however, the diameter of the arterial preparations was so small that there was not evident displacement, and the contractions were therefore essentially isometric.)

In all experiments the muscles were initially loaded with 1 g.

Physiological solutions

The smooth muscle polarizing solution (modified Krebs bicarbonate) comprised (mM): NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1·2, $CaCl_2$ 1·25 (half the usual amount) and glucose 11·5. Calcium-free polarizing solution was similar, but without the $CaCl_2$.

The depolarizing solution comprised (mM): NaCl 17, KCl 100, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1·2 and glucose 11·5 ; $CaCl_2$ was added according to the concentration required.

Drugs

Cinnarizine (Janssen Pharmaceutica) and chlorpromazine (Specia) were dissolved in physiological solution to the required concentrations. Adrenaline was administered as the bitartrate salt. The chelating agent ethylene diamine tetra-acetic acid (EDTA) was used to remove free calcium ions from solution.

Experimental procedures

Effect of calcium on the contraction of depolarized arterial smooth muscle

The calcium dose-effect relationship was investigated both by successive individual experiments at progressively higher calcium dosages (single dose technique) and by cumulative increments in the calcium concentration of the bath fluid (cumulative dose technique). For both techniques the $CaCl_2$ concentrations tested were 0·05, 0·1, 0·5, 1·25, 2·5, 5, 10 and 20 mM.

In the single dose experiments, the perfusion sequence was as follows: Ca-free Krebs bicarbonate + 2×10^{-4}M EDTA (5–10 min: until complete inhibition of the contractile response to depolarization was just achieved); Ca-free depolarizing solution + $CaCl_2$ to required concentration (3–5 min); Ca-free Krebs bicarbonate (wash, then 10 min); re-chelation and repetition of the experiment at the next $CaCl_2$ concentration.

In the cumulative dose experiments, the $CaCl_2$ additions were made one after the other as soon as a plateau response had been obtained at the previous concentra-

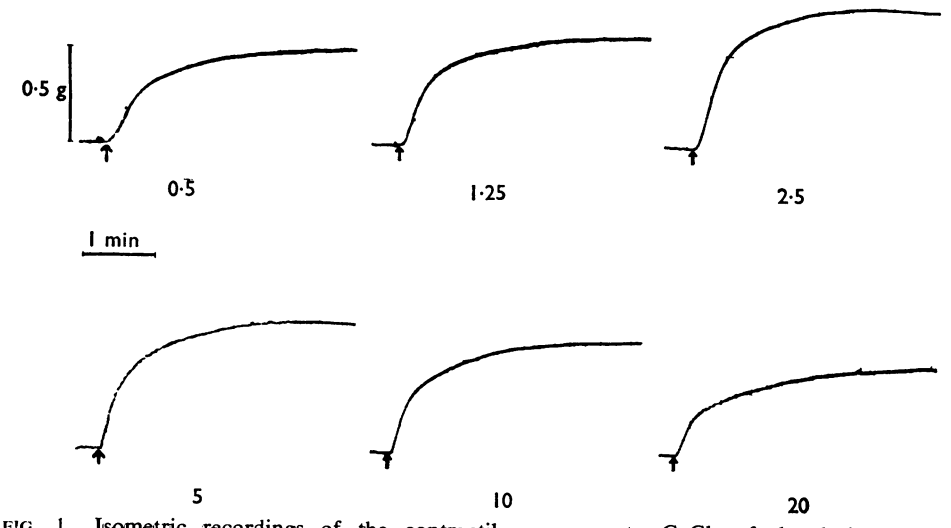

FIG. 1. Isometric recordings of the contractile response to $CaCl_2$ of depolarized rabbit mesenteric arteries of outside diameter 0·4–0·7 mm. Contractile tension (vertical axis) is plotted against time (horizontal axis). The mM concentration of $CaCl_2$ added at ↑ is given below each graph.

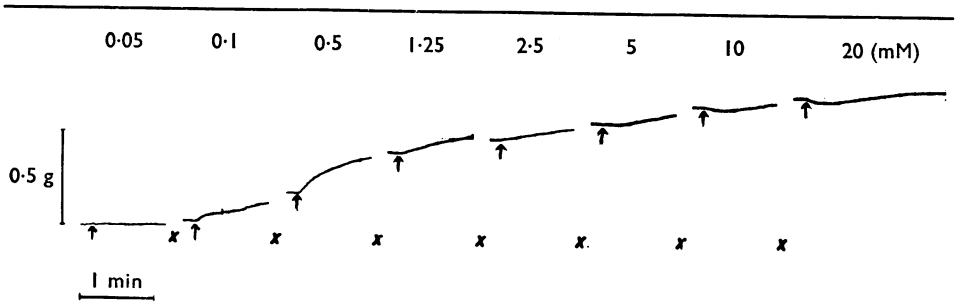

FIG. 2. Isometric recordings of the contractile response to cumulative increments in the $CaCl_2$ concentration of depolarized rabbit mesenteric artery of outside diameter 0·7 mm. The mM concentration of $CaCl_2$ added to the depolarizing solution at ↑ is given above each graph. × = 3 min.

tion ; the time interval between such successive calcium additions was usually 3–4 min, as compared with about 25 min for the single dose experiments.

Effect of cinnarizine on calcium-induced contraction of depolarized arterial smooth muscle

Antagonism of induction of contraction. In the single dose experiment, the perfusion sequence was as follows: modified Krebs bicarbonate + 10^{-6}M cinnarizine (90 min: until equilibrium reached) ; Ca-free Krebs bicarbonate + 10^{-6}M cinnarizine + 2×10^{-4}M EDTA (5–10 min: until complete inhibition of the contractile response to depolarization was just achieved) ; Ca-free depolarizing solution + 10^{-6}M cinnarizine + 20 mM $CaCl_2$; Ca-free Krebs bicarbonate (wash).

In the cumulative dose experiments, cinnarizine concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M were tested for the whole range of $CaCl_2$ doses.

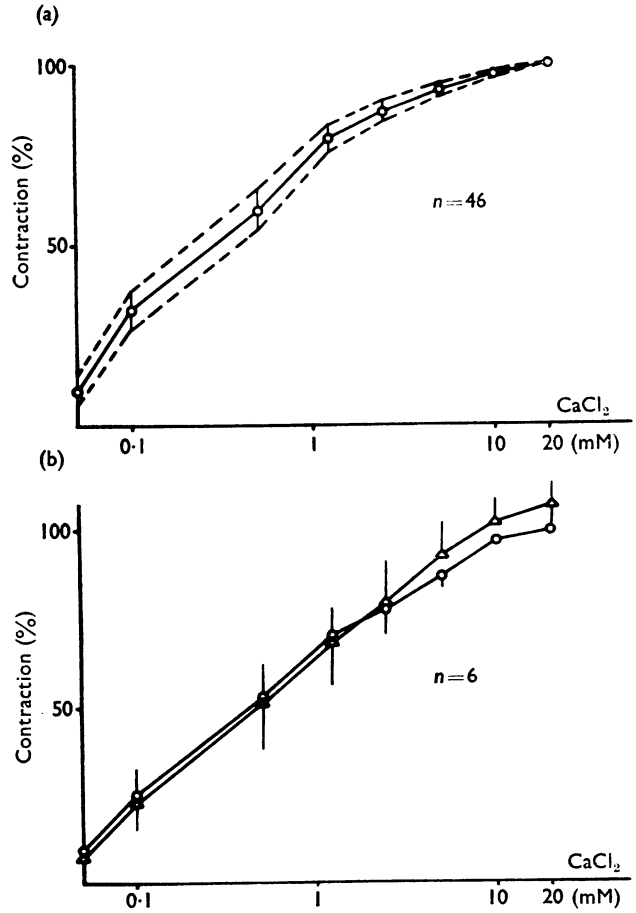

FIG. 3. Cumulative dose-effect curves of $CaCl_2$ on depolarized rabbit mesenteric arteries of outside diameter 0·4–0·8 mm. Contraction (as a percentage of the maximum response) is plotted against the $CaCl_2$ concentration in the depolarizing solution. In (*a*) the continuous line shows mean values of forty-six experiments and the broken line shows the confidence limits ($t \times$ S.E.). Graph (b) shows the reproducibility of the dose-effect curve: the time at rest in modified Krebs bicarbonate between the first experiment (○) and the second (△) was 90 min. Each point represents the mean of six experiments.

Before both single dose and cumulative dose experiments, a control curve was established for each preparation.

Relaxation of established contractions. Single dose experiments only were performed. After induced contraction of the preparation with 20 mM $CaCl_2$, the effect of cinnarizine (10^{-8}, 10^{-7} and 10^{-6}M) on the contractile tension was recorded, 5, 10, 15, 30, 45, 60, 75 and 90 min after addition of the antagonist.

Effect of cinnarizine on adrenaline-induced contraction of depolarized and of polarized arterial smooth muscle

Cinnarizine reduces the maximum response of mesenteric arteries to adrenaline (Godfraind *et al.*, 1968). Its effect was here investigated further by studying its effect at 10^{-6} and 10^{-5}M concentrations on the contraction induced by 10^{-4}M adrenaline, both in depolarizing solution and in polarizing solution (Krebs bicarbonate including $CaCl_2$).

Single dose experiments were performed on 1 mm diameter arteries and the perfusion sequence was as follows: Ca-free Krebs bicarbonate solution + 2×10^{-4}M EDTA (until complete inhibition of contraction to depolarization was just achieved: further prolongation reduced or abolished the subsequent response to adrenaline); Ca-free depolarizing solution addition of 10^{-4}M adrenaline; Krebs bicarbonate (30 min); addition of 10^{-4}M adrenaline; Krebs bicarbonate + cinnarizine (90 min); Ca-free depolarizing solution + 2×10^{-4}M EDTA (duration as for previous chelation); addition of 10^{-4}M adrenaline; Krebs bicarbonate (30 min); addition of 10^{-4}M adrenaline.

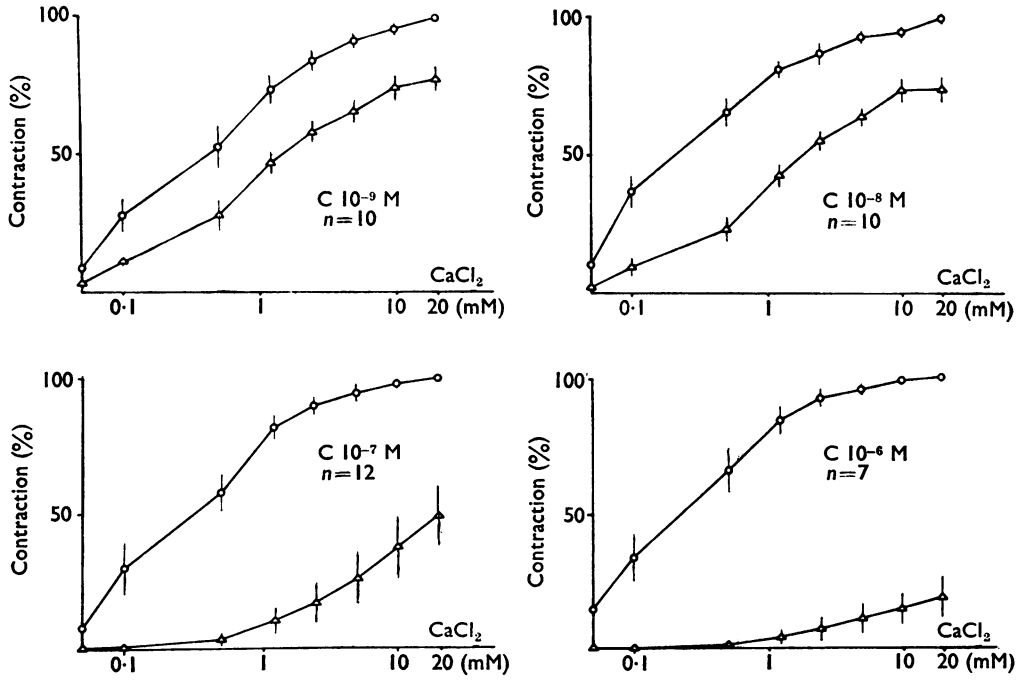

FIG. 4. Dose-effect curves of $CaCl_2$ on depolarized rabbit mesenteric artery of outside diameter 0·4–0·8 mm in the absence of cinnarizine (○) and 90 min after its addition to the medium (△).

Effect of chlorpromazine on arterial smooth muscle contraction

Experiments similar to those described above for cinnarizine were performed.

Results

Unless otherwise stated, the contractile response of the arterial smooth muscle preparation is expressed throughout as a percentage of the maximum contraction evoked by calcium or adrenaline.

Effect of calcium on the contraction of depolarized arteries

Calcium evoked sustained muscular contraction when added to the Ca-free depolarizing perfusate, and the magnitude of this response was dependent upon the $CaCl_2$ concentration in the bath fluid (Fig. 1). Contraction was abolished when the calcium was washed out of the preparation.

The shape of the contraction curves varied slightly with the assay technique: in the single dose experiments the $CaCl_2$ concentration giving maximum contraction was 2·5 mM (for example, Fig. 1) to 10 mM, according to the diameter of the vessel used, whereas in the cumulative dose experiments the $CaCl_2$ concentration giving the

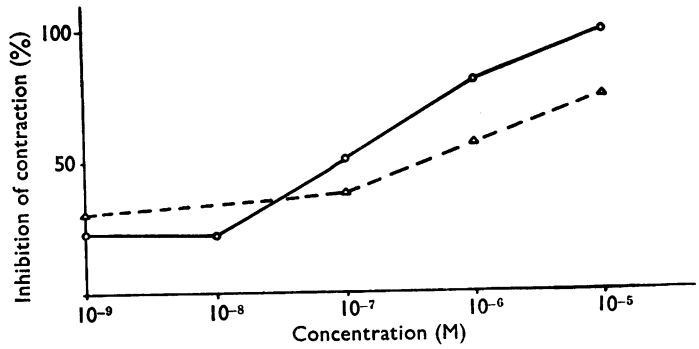

FIG. 5. Log dose-effect curves of cinnarizine (○) and chlorpromazine (△) as antagonists of the contraction of depolarized rabbit mesenteric arteries (outer diameter 0·4–0·8 mm) induced by 20 mM $CaCl_2$. Each value is the mean of at least six determinations for cinnarizine and three for chlorpromazine.

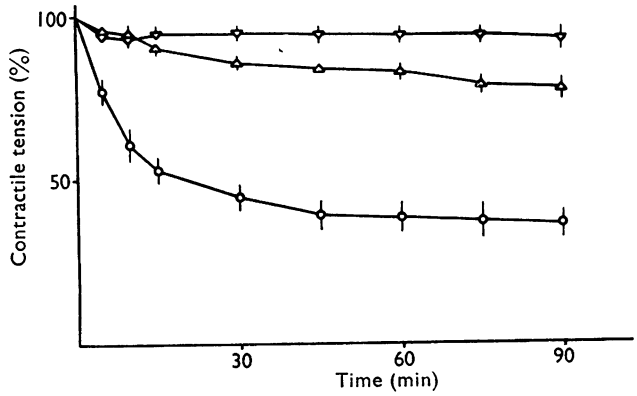

FIG. 6. Iscmetric tension changes of depolarized rabbit mesenteric arteries (outer diameter 0·7 mm) contracted by 20 mM $CaCl_2$, after addition of cinnarizine (▽——▽, 10^{-8}M, $n=3$; △——△, 10^{-7}M, $n=3$; ○——○, 10^{-6}M, $n=6$). Contractile tension (as a percentage of the maximum response) is plotted against incubation time after addition of cinnarizine.

maximum contraction was 10–20 mM (Figs. 3 and 4). In the cumulative dose experiments, with the smallest vessels (outer diameter 0·4–0·7 mm) the contraction in response to the highest calcium concentrations was preceded by a slight and transient relaxation (Fig. 2); at a $CaCl_2$ concentration of 50 mM, the contraction was below maximum; however, this concentration was little studied because of the hypertonicity of the perfusate.

The reproducibility of the contraction curves also varied with the assay technique: the cumulative dose experiments were readily reproducible when the time at rest in Krebs bicarbonate between two successive experiments was 90 min (Fig. 3b), but the single dose experiments gave no reproducible results.

To preclude as far as possible any differences in the results due to differences between the methods, the 20 mM $CaCl_2$ dosage and the 90 min time interval between successive experiments were adopted for subsequent single dose studies. In such conditions, at $CaCl_2$ concentrations of 20 mM, the magnitude of the response in both types of experiments was approximately equal.

Effect of cinnarizine on calcium-induced contraction of depolarized arterial smooth muscle

Antagonism of induction of contraction. Cinnarizine reduced the magnitude of the calcium-induced contraction of depolarized arterial smooth muscle at concentrations down 10^{-9}M. Increasing cinnarizine dosage reduced the magnitude of the maximum response and displaced the sigmoid log dose-effect curve to the right (Fig. 4). At 10^{-5}M concentration, cinnarizine completely abolished the contractile response.

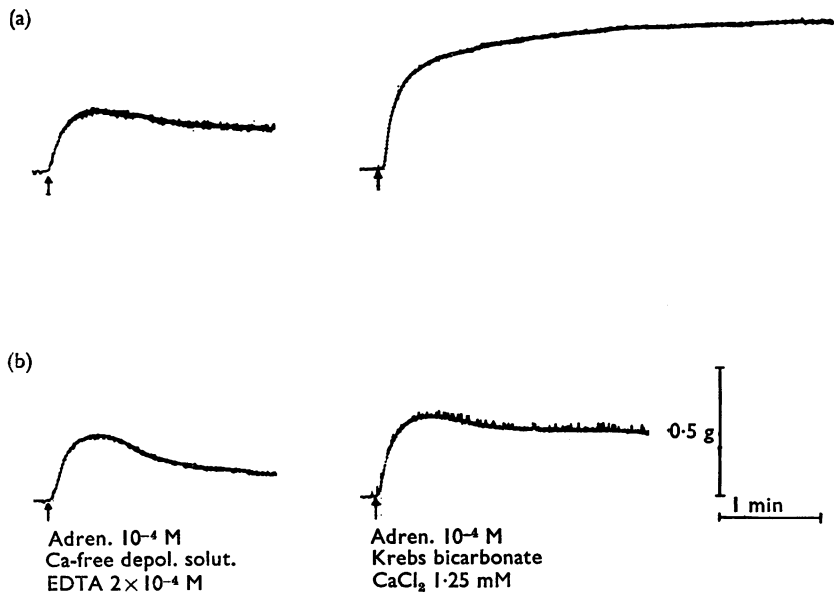

FIG. 7. Isometric recordings of the contractile response to adrenaline of rabbit mesenteric artery (outer diameter 1 mm) in calcium-free depolarizing solution or in Krebs bicarbonate polarizing solution. Upper graphs (a) show the control responses and lower graphs (b) show the responses after incubation for 90 min in Krebs bicarbonate containing 10^{-5}M cinnarizine.

In cumulative dose experiments using 0·4–0·8 mm diameter vessels, the maximum residual response to 20 mM $CaCl_2$ when treated with 10^{-7}M cinnarizine was only 50% of the maximum control value (Fig. 5) and, when treated with 10^{-6}M cinnarizine, only $19 \pm 8\%$ ($n=7$). Using 1 mm diameter vessels the inhibitory effect of cinnarizine was less marked: at the same concentrations (20 mM $CaCl_2$, 10^{-6}M cinnarizine) as those used for the 0·4–0·8 mm diameter vessels, the residual response was 60% ($n=3$, cumulative dose experiments).

Relaxation of established contraction. For arterial smooth muscle previously contracted by treatment with $CaCl_2$, cinnarizine decreased the contractile tension, and this relaxant effect increased with time and with increasing cinnarizine dosage (Fig. 6). The residual muscle tension after incubation for 90 min with a given concentration of cinnarizine was approximately equal to that achieved by antagonism of the induction of contraction after preincubation with cinnarizine for 90 min: using 0·7 mm diameter vessels, 20 mM $CaCl_2$ and 10^{-6}M cinnarizine, previously induced calcium contractions were relaxed, giving a residual response of 37% of the control value, and after washing out and re-evoking calcium-induced contractions in the presence of cinnarizine, the tension developed by the muscle was 41% of the control value.

The rate of action of cinnarizine was also similar for both induction of relaxation and antagonism of contraction: in the same circumstances (0·4–0·7 mm diameter vessels, 20 mM $CaCl_2$ and 10^{-6}M cinnarizine), 50% of the maximum relaxation of

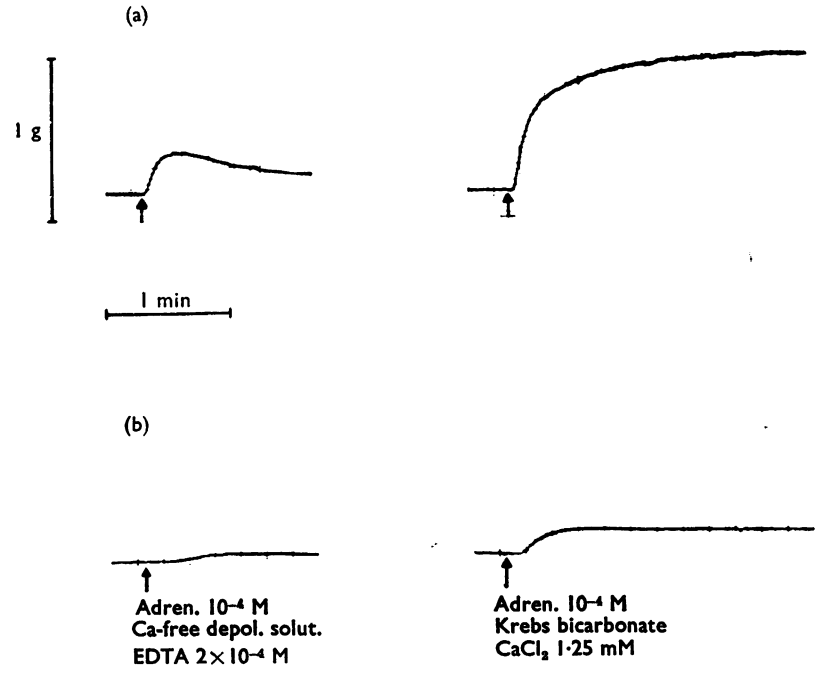

FIG. 8. Contractile response to adrenaline of rabbit mesenteric artery before (upper graphs) and after (lower graphs) treatment with 10^{-6}M chlorpromazine instead of 10^{-5}M cinnarizine. Other details are as in Fig. 7.

previously contracted muscle was developed in 13 min (Fig. 6), and 50% inhibition of the maximum contraction of previously relaxed muscle was developed in 16 min ($n=6$).

Effect of cinnarizine on adrenaline-induced contraction of depolarized and of polarized arterial smooth muscle

Adrenaline evoked marked smooth muscle contraction in the presence of calcium (Krebs bicarbonate) and a smaller, but definite, response in the absence of calcium (depolarizing solution + EDTA 2×10^{-4}M (Table 1 and Fig. 7). Treatment with cinnarizine reduced the magnitude of contraction in the presence of calcium but not in its absence (Fig. 7). Furthermore, after treatment with cinnarizine, the adrenaline-induced contraction was approximately as weak in the presence as in the absence of calcium (Fig. 7).

Effect of chlorpromazine on arterial smooth muscle contraction

Chlorpromazine antagonized calcium-induced contraction of depolarized arterial smooth muscle; a 4×10^{-7}M concentration of chlorpromazine caused 50% reduction in the contraction evoked by 20 mM $CaCl_2$ (Fig. 8) and a 10^{-4}M concentration caused complete abolition of the response. (In this respect cinnarizine was more potent than chlorpromazine, giving 50% reduction at 10^{-7}M and complete abolition at 10^{-5}M concentration.) The magnitude of the calcium-induced contraction after chlorpromazine incubation for 90 min was approximately the same whether the drug was inducing relaxation of previously contracted muscle or antagonizing contraction of previously relaxed muscle.

Chlorpromazine antagonized adrenaline-evoked contraction in the presence of calcium, and in this respect it was 100 times more potent than cinnarizine (Table 1). Unlike cinnarizine, however, it also antagonized the action of adrenaline in the absence of calcium in the solution and in the presence of 2×10^{-4}M EDTA. In both cases the antagonism by chlorpromazine was dose-dependent (Table 1).

TABLE 1. *Contractile response of rabbit vascular smooth muscle to* 10^{-4}M *adrenaline in the presence and absence of antagonists*

Muscle polarized: + calcium + adrenaline − antagonist	Muscle depolarized: − calcium + adrenaline − antagonist	Antagonist	Concentration of antagonist	Muscle depolarized: − calcium + adrenaline + antagonist	Muscle polarized: + calcium + adrenaline + antagonist	No of experiments
100	28·8±4·1	—	0 (control)	24·1±4·4	109±3·1	5
100	23·9±3·2	Cinnarizine	10^{-6}M	22·9±2·7	69±2·5	11
100	32·8±3·7	Cinnarizine	10^{-5}M	29·5±4·6	48±3·9	5
100	26 ±5·3	Chlorpromazine	10^{-8}M	22·0±2·7	106±3·7	4
100	31 ±3·0	Chlorpromazine	10^{-7}M	12·3±5·3	54±3·1	3
100	31·9±1·8	Chlorpromazine	10^{-6}M	4·5±1·1	26±5·9	3

1 mm diameter mesenteric arteries assayed; polarizing solution=Krebs bicarbonate ($CaCl_2$=1·25 mM); depolarizing solution = potassium-enriched, calcium-free Krebs bicarbonate + 2×10^{-4}M EDTA: duration of incubation of preparations with antagonist=90 min. Figures in columns 1, 2, 5 and 6 indicate mean % (± standard error) of the isometric contractile response evoked by 10^{-4}M adrenaline in the initial polarizing solution in the presence of calcium and the absence of antagonists.

Discussion

Effect of calcium on the contraction of depolarized smooth muscle

Calcium produces graded and reversible contractions of depolarized rabbit mesenteric arterial smooth muscle. A similar action of calcium has been recorded for the smooth muscle of guinea-pig taenia coli (Durbin & Jenkinson, 1961) and rat uterus (Edman & Schild, 1962). Comparison of the experimental results reported here with those of the authors cited above shows that there are only slight quantitative differences between the responses of the smooth muscle from the three sites. The threshold dose of calcium producing a contraction was 0·05 mM for rabbit mesenteric artery, 0·1 mM for rat uterus and 2 mM for taenia coli. Maximal contraction of rat uterus occurred at a concentration of 1 mM, of guinea-pig taenia coli at 6 mM, and of rabbit mesenteric artery at 2·5–20 mM (depending on the technique and the diameter of the artery). As found for rat uterus (Edman & Schild, 1962), doses above that required to obtain maximum contraction had an auto-inhibitory effect on the response of rabbit artery preparations.

Effect of cinnarizine and chlorpromazine on calcium-induced contraction of arterial smooth muscle

Cinnarizine and chlorpromazine both reduced the calcium-induced contraction of depolarized arteries. The degree of this reduction varied with the dose and the size of the vessels, 0·4–0·7 mm diameter arteries being more sensitive to such action than those of 1 mm diameter, a finding in agreement with earlier results (Godfraind *et al.*, 1968).

Site and mechanism of action of cinnarizine

The two most likely sites for cinnarizine receptors in arterial smooth muscle would seem to be the membrane of the muscle fibril (where the drug would reduce the availability of calcium ions to the contractile machinery of the depolarized muscle) or the contractile machinery itself (where it would interfere with the binding of calcium). As cinnarizine not only inhibits contractions evoked by calcium but also relaxes previously contracted muscle, two different mechanisms may be involved. However, whether its calcium antagonism is expressed by relaxing a given preparation or preventing its contraction, cinnarizine has the same time course for onset of action and the same magnitude of effect. In fact, therefore, it seems probable that its mechanism of calcium-antagonism in both situations is the same.

As cinnarizine does not modify the contraction evoked by adrenaline in calcium-free depolarizing solution, it is unlikely that it acts directly on the contractile proteins. The present results therefore favour the idea of localized action by cinnarizine on the cell membrane, such that the availability of free Ca^{++} ions to the contractile machinery is reduced.

Reduction in the availability of free Ca^{++} ions by accumulation of calcium has been postulated by Schild (1967) to explain the antagonism of calcium-induced contraction of depolarized rat uterus by isoprenaline. Recent findings of Feinstein, Paimre and Lee (1968) are in agreement with Schild's hypothesis. However, the mechanism of action of isoprenaline may be different from that of cinnarizine and it is not possible at present to decide whether cinnarizine causes internal calcium

accumulation. The alternative is that it reduces the permeability of the depolarized membrane to Ca^{++} ions, thereby inhibiting the influx of these ions into the muscle cell; free Ca^{++} ions in the cell are depleted by accumulation (Schild, 1967), extrusion (Hasselbach, 1964) or some other process or processes; the availability of free Ca^{++} ions for participation in the contraction process decreases and, consequently, calcium-induced muscle tension gradually weakens or is progressively less easily developed. If this hypothesis is correct, the polyvalent antagonism of cinnarizine is explicable in terms of a single mechanism.

Site and mechanism of action of chlorpromazine

Chlorpromazine antagonizes the α effects of adrenaline (Ariens & Simonis, 1964) and the calcium-induced contraction of depolarized smooth muscle (present experiments). These effects of chlorpromazine must involve two distinct mechanisms of action as there are drugs that can produce the one effect but not the other; for example, cinnarizine inhibits calcium-induced contraction of KCl-depolarized muscle but has no α-blocking activity (Van Nueten, Dresse & Dony, 1964), whereas phentolamine is an effective α-blocking agent but does not reduce contraction of KCl-depolarized muscle (Shibata & Carrier, 1967).

In polarized arterial smooth muscle, adrenaline probably increases both membrane permeability to extracellular calcium ions and intracellular mobilization of sequestered calcium (Bohr, 1965, 1967; Daniel, 1964, 1965; Hudgins & Weiss, 1968; Somlyo and Somlyo, 1968; Sullivan & Briggs, 1968). In the muscle depolarized in a Ca-free medium it probably increases only the mobilization of sequestered calcium. The present results are compatible with such a hypothesis, and it seems possible that chlorpromazine antagonizes both the increase in permeability to Ca^{++} ions and the mobilization of sequestered calcium, but that cinnarizine antagonizes selectively the increase in membrane permeability to Ca^{++} ions.

The mode of action of chlorpromazine on calcium activity in the smooth muscle studied may be different from its mode of action on calcium activity in striated muscle (Bazler & Makinose, 1968), as other drugs are known to affect these systems differently. For example, Scales & McIntosh (1968) have shown that phentolamine is more potent than phenoxybenzamine as an inhibitor of the uptake of calcium ions by sarcoplasmic reticular fractions, whereas the latter drug depresses vessel contraction in response to depolarization and the former does not.

One of us (A. K.) is Congo fellow of the O.C.D. (Belgium). We are grateful for the gift of chlorpromazine (Specia-Belgium) and of cinnarizine (Dr. Paul Janssen, Janssen Pharmaceutica, Beerse, Belgium), and we thank Dr. A. E. F. Chandler for his help in the preparation of the manuscript.

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(*Received February* 5, 1969)