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Effects of chlorbutol on ^{45}Ca movements and contractile responses of rat aorta and its relevance to the actions of Syntocinon

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The effects of chlorbutol (0·7, 1·4 and 2·8 mM) on the contractile responses induced by KCl and noradrenaline (NA) and on ^{45}Ca movements have been studied on rat isolated thoracic aorta. Chlorbutol decreased, in a dose-dependent manner, contractions induced by KCl and NA and this effect was observed whether it was added before or after the induced contractions. Preincubation with chlorbutol inhibited the contractile responses elicited by addition of Ca (1–5 mM) to Ca-free high-potassium solution. It also inhibited in a dose-dependent manner the ^{45}Ca influx but increased ^{45}Ca efflux in rat aortic strips. These results suggest that chlorbutol decreases peripheral resistance by reducing the availability of intracellular Ca to the contractile machinery in vascular smooth muscle cells. The effects of synthetic oxytocin (Syntocinon) at concentrations containing the same chlorbutol concentration were quantitatively similar from those produced by chlorbutol alone. Therefore, the inhibitory cardiovascular effects ascribed previously to synthetic oxytocin may be attributed to its preservative, chlorbutol, and not to oxytocin itself.

Although the effects of synthetic oxytocin (Syntocinon) on the uterus has been well established, its action on vascular smooth muscle is less well understood (Nakano 1974). Thus, in the rat some studies demonstrate that Syntocinon produced no significant change in systemic blood pressure (Lloyd 1959a, b), others found a hypertensive response (Rudinger et al 1972; Nakano 1974). The underlying haemodynamic mechanism for this discrepancy remains uncertain, since Syntocinon decreases total peripheral resistance in several vascular beds of the rat (Berde 1965; Goldman 1968).

Barrigón et al (1980) have reported that Syntocinon inhibits the contractions induced by different agonists and that it reduces the ^{45}Ca influx and increases the ^{45}Ca efflux in the rat isolated thoracic aorta. However, some of the effects of Syntocinon have been attributed not to oxytocin but to the vehicle and more specifically to the chlorbutol (chlorobutanol) (Katz 1964; Botting 1981). Chlorbutol, the preservative in Syntocinon is pharmacologically active and it has been used as an oral hypnotic in man (Sollman 1957). It also produces local anaesthesia, hypotension and a decrease in the respiratory centre response to carbon dioxide (Sollman 1957). Therefore we have determined the effects of chlorbutol on drug-induced contractions of rat isolated thoracic aortic strips, and Ca movements in isolated arterial smooth muscle cells, in order to compare the effects produced by chlorbutol with those produced by a concentration of Syntocinon containing the same concentration of chlorbutol.

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MATERIALS AND METHODS

Experimental procedure

Sprague-Dawley rats (200–250 g) were killed by a blow on the head. The thoracic aorta was removed and helically cut strips prepared and mounted as described by Furchgott & Bhadrakom (1953). Strips (0·3 × 2–3 cm) were tied at both ends and suspended in 10 ml organ baths containing Krebs-bicarbonate solution (KBS) of the following composition (mM): NaCl 118, KCl 4·75, KH_2PO_4 1·2, $NaHCO_3$ 25, $CaCl_2$ 1·8, $MgSO_4$ 1·2, glucose 11. Calcium-free KBS contained no calcium chloride but 0·1 mM disodium dihydrogen ethylenediamine tetracetate (Na_2EDTA) was added. Solutions were aerated with 95% 0_2 – 5% CO_2. Bath temperature was maintained at 34 °C. The amplitude of aortic contractions was measured isometrically by a Grass FT 03 force-displacement transducer and recorded on a linear recorder (Riken-Denshi F43) coupled to a Grass polygraph. Strips were allowed to equilibrate for 2 h under 2 g tension before addition of drugs. After 2 h equilibration the following experiments were performed.

In the first group, aortic strips were exposed to a single dose of noradrenaline (NA) (1 μM) or potas-

sium chloride (80 mM). The concentrations selected for each agonist produced submaximal contractions of the strips and only one agonist was used in each experiment. Control responses for each agonist were obtained at 30 min intervals by exposure to the agonist at the beginning of the experiment until two successive contractile responses were almost identical in height. After the agonist had been removed by washing, the strips were exposed to the desired concentration of chlorbutol or Syntocinon for 5 min, the agonist was re-added to the bath and the contraction was recorded. The values of the contractile responses obtained in the presence of these compounds were expressed as percentage of the control contractions in each experiment.

In another group of experiments to determine whether chlorbutol and Syntocinon could relax already established contractions, aortic strips were contracted by single submaximal doses of NA or KCl. When the contractile responses to either agonist was maximum, chlorbutol or Syntocinon were added, in progressively increasing cumulative doses.

In additional experiments, aortic strips were equilibrated in calcium-free KBS for 2 h with successive washings in this solution every 20 min. After equilibration, strips were exposed to calcium-free high potassium (80 mM) depolarizing KBS for 5 min and then calcium (1–5 mM) was added to the bath in stepwise fashion over the next 50 min (Tamargo et al 1978). Calcium was then washed out and strips reincubated in calcium-free KBS for 60 min. The high potassium depolarizing procedure was repeated, but 5 min before the first addition of calcium, chlorbutol or Syntocinon were added to the bath.

^{45}Ca influx and ^{45}Ca efflux

Measurements of ^{45}Ca influx and ^{45}Ca efflux were also undertaken using the techniques of Van Breemen et al (1972). In the experiments the aorta was bisected and each half mounted in a separate organ bath containing Tris-buffered solution (TS) of the following composition (mM): NaCl 160, KCl 4·6, $CaCl_2$ 1·5, $MgCl_2$ 1·0, glucose 11 and Tris 6·0. Solutions were adjusted to pH 7·4 with 4·0 M HCl and aerated with 100% O_2.

To determine ^{45}Ca influx, aortic strips were placed under 2 g tension in TS for 2 h. Then they were exposed to TS-80 mM KCl plus ^{45}Ca (specific activity 0·2 $\mu Ci\ ml^{-1}$; Radiochemical Centre, Amersham). Following the experimental periods, the strips were placed in calcium-free TS containing 2 mM La Cl_3 for 1 h to remove extracellular bound calcium. Then, the strips were removed, blotted on Whatman no. 42 filter paper and weighed. The strips were then placed in scintillation vials and 0·5 ml of Soluene-100 (Packard) added and the strips digested overnight at 50 °C. Radioactivity was assayed in a liquid scintillation counter (Intertechnique Model SL-3000) as previously described (Barrigón et al 1978, 1982). To determine ^{45}Ca efflux, the strips were labelled with ^{45}Ca (specific activity 1·0 $\mu Ci\ ml^{-1}$) for 2 h before being placed in calcium-free TS with 2 mM $LaCl_3$. Strips were then removed at varying times, blotted, weighed and prepared for counting as described above.

Drugs

The chemicals and drugs used were: synthetic oxytocin (Syntocinon, Sandoz), noradrenaline bitartrate (Sigma), potassium chloride (Merck), calcium chloride (Merck), chlorbutol (Chlorobutanol) (Sigma). Ascorbic acid (10^{-4} M) was added to each solution of noradrenaline, made up freshly every day. The concentrations for each chemical or drug are expressed as final concentrations in the bath.

Throughout the paper, results are expressed as mean ± s.e.m. for each experimental group. Statistical analyses were performed by means of Student's *t*-test and considered significant if $P < 0{\cdot}05$.

RESULTS

Synthetic oxytocin (Syntocinon) is a water solution containing (ml): synthetic oxytocin, 10 i.u., chlorbutol 0·5%, ethanol 0·61% by volume, sodium acetate 1 mg and acetic acid to pH 4 ± 0·3. In preliminary experiments we demonstrated that in the same range of concentrations chlorbutol, but not acetate buffer or ethanol, modified the resting tension or the contractile responses induced by NA or KCl in isolated aortic strips. Therefore, throughout the paper, the effects of Syntocinon will be referred to as effects of chlorbutol plus oxytocin.

Chlorbutol (0·7, 1·4 and 2·8 mM) and oxytocin (0·5, 1 and 2 μM) plus chlorbutol (0·7, 1·4 and 2·8 mM) produced a dose-dependent decrease of the contractile responses induced by single submaximal doses of NA or KCl in aortic strips. Fig. 1 shows recordings of typical changes of a 5 min incubation period at three different concentrations of chlorbutol on the contractile responses induced by both agonists. The greater the concentration of drugs, the more rapidly the inhibition became manifest. Since contractile responses to NA and KCl in isolated

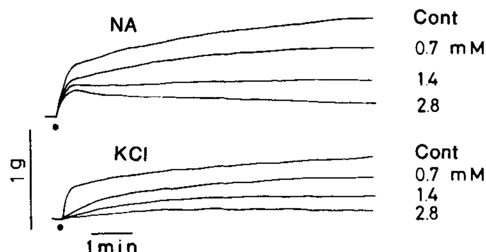

FIG. 1. Effects of chlorbutol (0·7, 1·4 and 2·8 mM), on the isometric contractions of rat aortic strips induced by noradrenaline (NA, 10^{-6} M) and potassium chloride (KCl, 80 mM). Chlorbutol was added to the bath 5 min before the addition of the agonist at (●).

aortic strips can be resolved into a fast initial and a slow late component (Godfraind & Kaba 1972), the contractile response induced by these agonists was fitted as the sum of a two exponential process,

$$C = C_1(1 - e^{-t/\tau_1}) + C_2(1 - e^{-t/\tau_2})$$

where C is the total contractile response at time t; C_1 is the amplitude of the fast contractile response and τ_1 its time constant; C_2 and τ_2 are the amplitude and the time constant, respectively of the slow contractile response to both agonists (Table 1).

Fig. 2 shows the effect of chlorbutol (0·7, 1·4 and 2·8 mM) and oxytocin (0·5, 1 and 2 μM) plus chlorbutol on the fast and slow components of the contractile responses induced by NA and KCl. At concentrations of chlorbutol ≤ 1·4 mM both compounds did not modify ($P > 0{\cdot}05$) the fast component of the NA-induced responses and the slow component of the KCl-induced responses. However, both compounds significantly ($P < 0{\cdot}05$) reduced in a dose-response manner the slow component of the NA-induced responses and the fast component of the KCl-induced responses. When chlorbutol concentration reached 2·4 mM, the two compounds significantly reduced ($P < 0{\cdot}05$) the fast component of the NA-induced responses and the slow component of the KCl-induced responses. At this concentration both the fast KCl component and the slow NA component were abolished. The effect of cumulative doses of chlorbutol and oxytocin plus chlorbutol

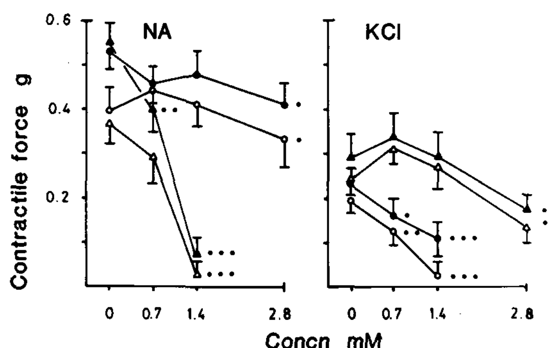

FIG. 2. Effects of chlorbutol (0·7, 1·4 and 2·8 mM) on fast (○) and slow (△) components and oxytocin (0·5, 1 and 2 μM) plus chlorbutol (0·7, 1·4 and 2·8 mM) on fast (●) and slow (▲) components of the isometric contractions induced by NA (left) and KCl (right) on rat isolated aortic strips. Ordinate: contractile force (g). Abscissa: drug concentration (mM). Each point represents the mean ± s.e. of 6 experiments. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

after the NA- and KCl-induced contractions reached their maximal height is illustrated in Fig. 3. Each muscle was used for only one test. Chlorbutol (0·7, 1·4, 2·8 mM) and oxytocin (0·5, 1 and 2 μM) plus chlorbutol (0·7, 1·4, 2·8 mM) consistently caused a dose-dependent relaxation of the contracted strips.

Table 1. Effect of Syntocinon and chlorbutol on the τ_1 and τ_2 values of the NA- and KCl-induced contractile responses.

	Drug concn		NA		KCl	
	mM	μM	τ_1 (s)	τ_2 (min)	τ_1 (s)	τ_2 (min)
Chlorbutol	0	—	6·17 ± 0·73	2·21 ± 0·10	7·30 ± 0·54	2·36 ± 0·21
	0·7	—	4·97 ± 0·46	1·83 ± 0·11*	18·20 ± 1·17***	1·95 ± 0·12
	1·4	—	4·96 ± 0·57	—	—	1·34 ± 0·11**
	2·8	—	5·42 ± 0·53	—	—	1·11 ± 0·13***
Oxytocin plus chlorbutol	0	0	6·31 ± 0·99	2·36 ± 0·18	5·83 ± 0·47	2·46 ± 0·13
	0·7	0·5	5·23 ± 0·39	1·94 ± 0·11*	12·74 ± 1·52**	2·23 ± 0·12
	1·4	1·0	5·81 ± 0·35	—	14·22 ± 1·61***	2·05 ± 0·10*
	2·8	1·5	7·31 ± 1·12	—	—	0·87 ± 0·20***

Mean ± s.e.m. values are given.
τ_1 = time constant of the fast contractile responses.
τ_2 = time constant of the slow contractile responses.
*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

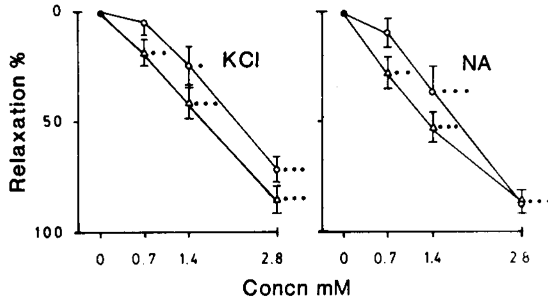

FIG. 3. Relaxation of KCl- and NA-induced contractions by chlorbutol (0·7, 1·4 and 2·8 mM) (△) and oxytocin (0·5, 1 and 2 μM) plus chlorbutol (0·7, 1·4, 2·8 mM) (○). Ordinate: relaxation (%). Abscissa: drug concentration (mM). Each point represents the mean ± s.e. of 6 experiments. $*P <$ 0·05, $**P <$ 0·01, $***P <$ 0·001.

Effect on calcium chloride-induced contractions on potassium-depolarized aortic strips

When aortic strips were exposed to calcium-free high potassium TS, addition of calcium to the bath produced a progressive increase in the developed tension. Chlorbutol (1·4 mM) and oxytocin (1 μM) plus chlorbutol shifted the concentration-response curve of the calcium chloride downwards and to the right (Fig. 4) and the maximal contraction induced by the stepwise increase of calcium to the bath was significantly reduced by both drugs ($P <$ 0·001). This observation suggested that both compounds might inhibit the inward movement of added calcium across the potassium-depolarized aortic strips.

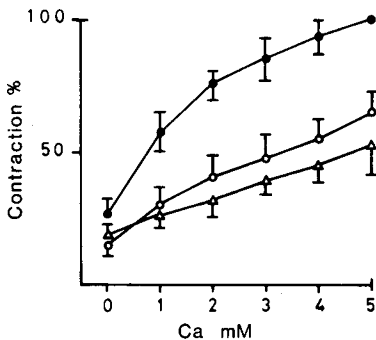

FIG. 4. Effect of chlorbutol and oxytocin plus chlorbutol on the isometric contractions elicited in aortic strips by addition of calcium (1–5 mM) to calcium-free high potassium (80 mM) medium. Ordinate: percentage of the maximum control contraction obtained with the highest concentration of calcium in each experiment. Abscissa: calcium concentration (mM). Both compounds were added to the bath 5 min before the addition of the high potassium medium. Each point represents the mean ± s.e. of 7 experiments. (●) Controls. (△) Chlorbutol, 1·4 mM. (○) Oxytocin, 1 μM, plus chlorbutol, 1·4 mM.

Effects of ^{45}Ca influx and ^{45}Ca efflux

The effect of two different concentrations of chlorbutol (1·4 and 2·8 mM) and oxytocin (1 and 2 μM) plus chlorbutol on ^{45}Ca influx and ^{45}Ca efflux was estimated at different times (5, 10, 15 and 30 min) in aortic strips. Fig. 5 shows that the ^{45}Ca influx in strips

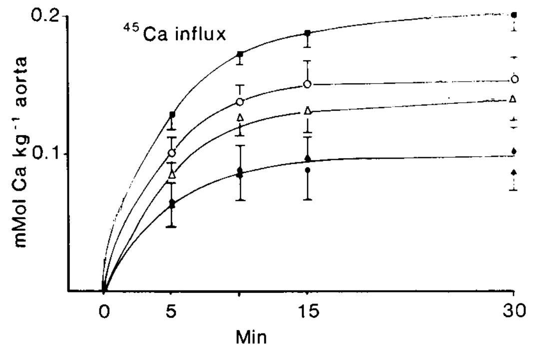

FIG. 5. Effect of chlorbutol and oxytocin plus chlorbutol on ^{45}Ca influx in aortic strips. After equilibration aortae were exposed to ^{45}Ca. After various times, strips were placed in calcium-free solution containing 2 mM $LaCl_3$ for 1 h. Ordinate: ^{45}Ca content in mmol kg^{-1} of aorta. Abscissa: time (min) in radioactive solution. Each point represents the mean ± s.e. of 6 experiments. (■) Controls. (△) Chlorbutol, 1·4 mM. (▲) Chlorbutol, 2·8 mM. (○) Oxytocin (1 μM) plus chlorbutol (2·8 mM). (●) Oxytocin (2 μM) plus chlorbutol (2·8 mM).

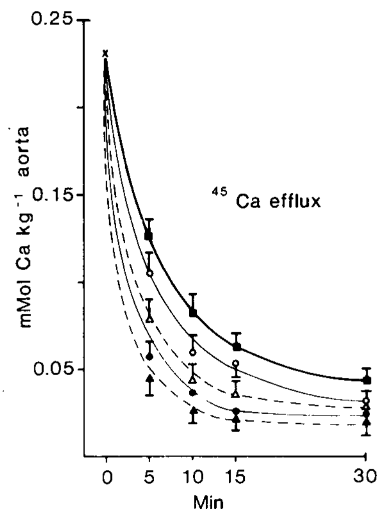

FIG. 6. Effect of chlorbutol and oxytocin plus chlorbutol on ^{45}Ca efflux from rat aortic strips. Aortae were exposed to ^{45}Ca for 2 h and then placed in calcium-free solution containing 2 mM $LaCl_3$. Ordinate: ^{45}Ca content in mmol kg^{-1} of aorta. Abscissa: time (min) in non-radioactive physiological solution. Each point represents the mean ± s.e. of 6 experiments. (■) Controls. (△) Chlorbutol, 1·4 mM. (▲) Chlorbutol 2·8 mM. (○) Oxytocin (1 μM) plus chlorbutol (1·4 mM). (●) Oxytocin (2 μM) plus chlorbutol (2·8 mM).

incubated in the presence of both compounds was significantly lower than the corresponding control values at all times ($P < 0{\cdot}05$) and that the effect was dependent on the concentration of both drugs in the organ bath. They also increased significantly ($P < 0{\cdot}05$) in a dose-dependent manner, the ^{45}Ca efflux at all times (Fig. 6). No significative differences were found on ^{45}Ca influx and ^{45}Ca efflux between chlorbutol and oxytocin plus chlorbutol.

DISCUSSION

The results of the present study indicate that chlorbutol inhibits, in a dose-dependent manner, the contractile responses induced by NA and KCl, decreases ^{45}Ca influx and increases ^{45}Ca efflux in rat aortic strips. Furthermore, the results show that the contractile responses induced by addition of calcium to a calcium-free high K-KBS are also inhibited. There are three sources of Ca: (a) extracellular Ca, (b) Ca which is loosely bound to the cell membrane, and (c) Ca which is firmly bound to the cell membrane or to the intracellular components (Hudgins & Weiss 1968; Van Breemen et al 1980). The potassium-induced response is initiated by a depolarization of the cell membrane and subsequent influx of extracellular and/or loosely bound Ca through voltage-dependent channels into the smooth muscle cell (Bolton 1979; Casteels 1980). An increase in Ca influx through a receptor-operated channel is thought to be responsible for the slow component of the NA-induced responses (Godfraind & Kaba 1972; Deth & Van Breemen 1977). On the other hand, the release of Ca from firmly bound intracellular stores seems to be the determinant of the fast component of the NA-induced responses and of the slow component of the KCl-induced responses (Hinke 1965; Hudgins & Weiss 1968; Goodman & Weiss 1971).

Chlorbutol ($\leqslant 1{\cdot}4$ mM) significantly inhibited contractions due to Ca influx, the fast component of the KCl-induced and the slow component of the NA-induced responses. These results suggest that at this range of concentrations chlorbutol may reduce the influx of Ca responsible for both contractile components. Another three experimental results lends further support to the above hypothesis: (1) chlorbutol shifted the dose-response curve to Ca to the right in high K-depolarized aortic strips. Addition of successive concentrations of Ca to the bath resulted in a dose-dependent increase in the developed tension of strips perfused with calcium-free high potassium KBS. High potassium depolarized the cell membrane and stimulated the influx of Ca through voltage-dependent channels, which may induce a contraction directly or by triggering further release of intracellular stored Ca (Briggs 1962; Van Breemen & McNaughton 1970; Bolton 1979). Since preincubation with chlorbutol consistently shifted the dose-response curve to Ca to the right, this inhibitory effect could be attributed to its ability to inhibit the influx and/or the availability of extracellular and superficial Ca in aortic smooth muscle cells. (2) Chlorbutol produced a dose-dependent decrease in ^{45}Ca influx at all time intervals which clearly indicates that they inhibit the influx of Ca from extracellular or from superficially loosely bound stores in aortic smooth muscle cells. (3) Calcium antagonists, e.g. verapamil, also abolished contractions due to Ca influx, Ca-induced contractile responses of KCl-depolarized vascular smooth muscle and inhibited ^{45}Ca influx (Fleckenstein 1977; Van Breemen et al 1980; Vanhoutte 1982).

At higher concentrations chlorbutol not only abolished these contractile responses but also inhibited the fast component due to NA and the slow component due to KCl. Since these latter components are due to release of Ca from intracellular stores, it seems reasonable to suggest that chlorbutol must exert also an intracellular effect in addition to its extracellular (or membrane) action discussed above. Furthermore, chlorbutol relaxes muscles which were already contracted by prior exposure to NA or KCl. This result again indicates that it could antagonize an intracellular step between Ca influx and the contractile elements. In fact, when the rate of Ca diffusing into the cell is slowed, the intracellular membranous relaxing system can remove a larger portion of the cytoplasmic Ca before it reaches the activation sites on the myofilaments (Van Breemen 1976). Therefore, in the presence of chlorbutol, the relaxing system might reduce significantly the access of Ca to the contractile elements or the release of Ca from intracellular stores. Furthermore, this drug increased ^{45}Ca efflux. Both effects would reduce the availability of intracellular Ca which could explain the reduction of the fast component due to NA and the relaxing effect of chlorbutol.

The effects of Syntocinon at concentrations containing the same chlorbutol concentration were indistinguishable from those produced by chlorbutol alone on both contractile responses and ^{45}Ca movements. This suggests that the inhibitory cardiovascular effects ascribed previously to synthetic oxytocin may have been due to its preservative, chlorbutol, contained in commercial preparations and not to

the oxytocin itself. Chlorbutol exhibits local anaesthetic properties (Sollman 1957) and inhibits the ^{45}Ca influx that could explain the vasodilator effect (Katz 1964; Nakano 1974) and the relaxing effect of smooth muscle in various organs (Somlyo & Somlyo 1968; Botting & Manley 1967). Since the activity of chlorbutol is far from negligible in vascular smooth muscle its presence in commercial preparations of oxytocin may be a source of error and this possibility should be emphasized.

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