J. Pharm. Pharmacol. 1991, 43: 411–416
Received October 29, 1990

Influence of Calcium Entry Blockers and Calmodulin Inhibitors on 5-Hydroxytryptamine-, Potassium- and Calcium-induced Contractions in Human Umbilical Artery In-vitro

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Abstract—The effects of calcium (Ca^{2+}) withdrawal, Ca^{2+} entry blockers and calmodulin inhibitors on the contractile responses to 5-hydroxytryptamine (5-HT), K^+ and $CaCl_2$ have been studied in human isolated umbilical artery. Following Ca^{2+} withdrawal from the medium (30 min), both 5-HT- and K^+ -induced contractions were virtually abolished. Ca^{2+} entry blockers (nifedipine, verapamil, diltiazem and cinnarizine) were significantly more effective in inhibiting 5-HT- than K^+-induced contractions. In relation to calmodulin inhibitors, trifluoperazine inhibited all types of contractions, whereas W-7 (*N*-6-aminohexyl-5-chloro-1-naphthalene sulphonamide) only inhibited 5-HT- and $CaCl_2$-induced contractions. All drugs inhibited $CaCl_2$-induced contractions in a non-competitive manner. These findings indicate that the mechanisms by which these drugs exert their inhibitory effects on human umbilical artery are markedly different from those reported in other vascular beds.

In spite of the great importance of human umbilical vessels before birth, the pharmacological knowledge about the mechanisms that modulate the contractile events of this tissue have been less studied in comparison with other vascular beds. Since umbilical vessels are not innervated (Hollingsworth 1974), it is likely that locally released substances such as 5-hydroxytryptamine (5-HT) (McGrath et al 1985), angiotensin II (Hosakawa et al 1985) and bradykinin (Altura et al 1983) may be involved in the control of vascular tone.

In this work we evaluated the effects of some calcium (Ca^{2+}) entry blockers (nifedipine, verapamil, diltiazem and cinnarizine) and the putative calmodulin inhibitors (trifluoperazine and W-7 (*N*-6-aminohexyl-5-chloro-1-naphthalene sulphonamide) (Asano & Hidaka 1985) on 5-HT- and K^+-evoked contractions of the isolated human umbilical artery. In addition, we also analysed the effect of these substances on Ca^{2+}-induced contractions in depolarized preparations.

Materials and Methods

The drugs used were: nifedipine, (±)- verapamil hydrochloride, cinnarizine, diltiazem hydrochloride, 5-hydroxytryptamine creatinine sulphate, trifluoperazine and W-7 (all from Sigma, USA). Stock solutions (1–10 mM) of these drugs were prepared as follows: verapamil, W-7 and trifluoperazine (water), nifedipine (absolute ethanol), cinnarizine and 5-HT (0·9% w/v NaCl solution containing HCl 0·01 M), stored at −4°C and diluted in water just before use. The experiments and solutions with nifedipine were protected from light and the organ chambers were covered with aluminium foil. The final ethanol bath concentration was less than 0·01% and did not interfere with the response to drugs.

Human umbilical cords were obtained from 32 normal deliveries at term pregnancies. Tissue samples were immediately transferred to Krebs solution (see composition below) and tested on the same day or stored at 4°C and studied on the next day. Umbilical artery segments from the placental end of the cord were carefully dissected from the surrounding tissues. In general, 4–6 rings (4–5 mm) obtained from the same cord were carefully suspended under a tension of 4 g during the equilibration period in a 5 mL organ bath, containing Krebs solution (mM: NaCl 118; KCl 4·4; $CaCl_2$ 2·5; $MgSO_4$ 1·1; $NaHCO_3$ 23·9; KH_2PO_4 1·1 and glucose 11·0, at 37°C, pH 7·2–7·4) gassed with 95% O_2-5% CO_2 dissolved in double distilled and demineralized water. Following the stabilization period (3–4 h), with the bath solution being changed every 30 min, tension was readjusted to 2 g and contractile responses for 5-HT, K^+ and $CaCl_2$ were recorded by means of a force transducer F-60 (Narco Biosystems). In experiments performed with 5-HT, EDTA (0·027 mM) was added to the Krebs solution to avoid oxidation of this monoamine. The high K^+ Krebs solution was prepared by equimolar replacement of NaCl with KCl (Calixto 1987).

To assess the participation of Ca^{2+} from extracellular sources on the contractile responses caused by either 5-HT (100 nM) or K^+ (80 mM), the preparations were initially contracted by a single concentration of these agonists at 60 min intervals. Once the contractile response became reproducible (in general, following 2 to 3 contractile responses), the Krebs solution was substituted by a similar solution, this time free of Ca^{2+}, and the contractile responses to 5-HT or K^+ were sequentially obtained at different intervals (3 and 30 min) following Ca^{2+} omission.

In a separate sequence of experiments following the equlibration period, the preparations were contracted by 5-HT (100 nM) or K^+ (80 mM), at 60 min intervals. After complete stabilization of the contractile responses to either 5-HT or K^+, different concentrations of nifedipine (0·1–1000 nM), verapamil (1–1000 nM), diltiazem (0·1–100 μM),

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cinnarizine (0·3–300 μM), trifluoperazine (0·03–300 μM) and W-7 (1–100 μM) were incubated in the bath for 30 min, except for cinnarizine (60 min), and another contractile response to 5-HT or K^+ was obtained. The mean of 2 to 3 initial control contractile responses to 5-HT or K^+ was expressed as 100% and all subsequent inhibitions were calculated as a function of this value. At least four different concentrations of the studied Ca^{2+} entry blockers or calmodulin inhibitors were tested in each preparation and only one compound was tested in each tissue. Since this preparation is a non-innervated tissue, the experiments performed in depolarizing solution (K^+ 80 mM) were carried out in the absence of an α-adrenoceptor antagonist.

In order to study the effect of Ca^{2+} entry blockers and calmodulin inhibitors on $CaCl_2$-induced contractile responses, the preparations were equilibrated for at least 120 min in normal Krebs solution and then exposed for about 1 h to high K^+, Ca^{2+}-free depolarizing solution (by equimolar replacement of 80 mM of NaCl by 80 mM of KCl). After this period, and following the complete relaxation of the preparations, contractile cumulative concentration-response curves (Van Rossum 1963) for $CaCl_2$ (0·1 to 100 mM) were constructed at 60 min intervals, in the absence or in the presence of different concentrations of nifedipine (0·003–0·01 nM), verapamil (3–10 μM), cinnarizine (10–30 μM), diltiazem (10–30 μM), trifluoperazine (100–300 μM) and W-7 (10–300 μM) which, with the exception of cinnarizine (incubated 60 min before), were incubated 30 min before in high K^+, Ca^{2+}-free solution. In these experiments, the organ bath concentration of $CaCl_2$ was increased by a factor of 3·2 and each concentration was added after the effect of the previous concentration had reached its maximum and had remained constant. The first cumulative concentration-response curve for $CaCl_2$ was taken as control. Only one compound was tested in each preparation and parallel control experiments were performed using only $CaCl_2$ to correct possible variation in the sensitivity of the preparation. Tissue sensitivities for all agonists were evaluated at the IC50 or ED50 levels (concentration of compound producing 50% of contraction or inhibition, respectively).

In another set of experiments, following the equilibration period in normal Krebs solution for at least 3–4 h, the preparations were maintained in high K^+, Ca^{2+}-free depolarizing solution (60 min) and were contracted by a single $CaCl_2$-concentration. After complete stabilization of the sustained Ca^{2+}-induced contraction (about 30 min), differ-

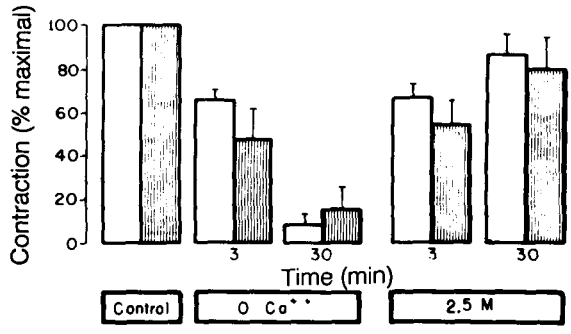

FIG. 1. Time course of the inhibitory effect of calcium-free medium on K^+ (80 mM) (□) and 5-HT (100 nM) (■)-induced contractions of human isolated umbilical artery. Ca^{2+} (2·5 mM) was subsequently re-added. Each column represents the mean of 5–6 experiments and the vertical bars the s.e.m.

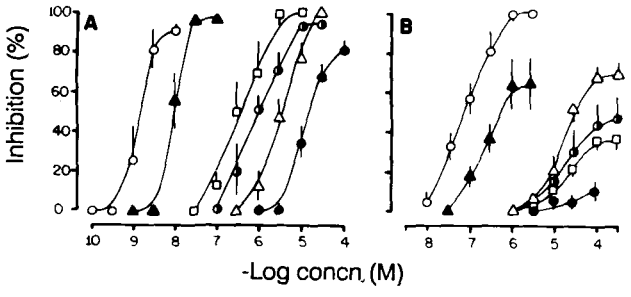

FIG. 2. Concentration response curves for the relaxant effects of nifedipine (○), verapamil (▲), diltiazem (□), cinnarizine (◑), trifluoperazine (△) and W-7 (●) in human umbilical artery contracted by 5-HT (100 nM) (A) and K^+ (80 mM) (B). Each point represents the mean of 6 experiments and the vertical lines the s.e.m.

Table 1. Potencies of calcium entry blockers and calmodulin inhibitors of the human isolated umbilical artery contracted by 5-HT (100 nM), K^+ (80 mM) and $CaCl_2$ (0·1-100 mM).

	IC50			
Drugs	5-HT	K^+	$CaCl_2$	K^+/5-HT[a]
Nifedipine	$1·0 \times 10^{-9}$ (0·8–12·0)[b]	$4·8 \times 10^{-8}$ (2·6–7·2)	$6·3 \times 10^{-12}$ (2·6–9·7)	48* (3·2–9·7)
Verapamil	$7·5 \times 10^{-9}$ (6·0–11·0)	$3·2 \times 10^{-7}$ (1·5–6·7)	$3·0 \times 10^{-6}$ (1·6–10·5)	42·7*
Diltiazem	$8·5 \times 10^{-7}$ (6·0–12·7)	$2·0 \times 10^{-5}$ (1·8–5·0)	$8·6 \times 10^{-5}$ (5·2–13·0)	23·5*
Cinnarizine	$2·8 \times 10^{-6}$ (1·2–8·0)	$1·2 \times 10^{-5}$ (0·8–1·8)	$1·7 \times 10^{-5}$ (1·2–1·9)	4·3*
Trifluoperazine	$4·1 \times 10^{-7}$ (3·5–8·0)	$3·8 \times 10^{-5}$ (1·3–9·0)	$1·5 \times 10^{-4}$ (0·9–3·0)	92·6*
W-7	$1·5 \times 10^{-5}$ (0·9–2·3)	c	$1·3 \times 10^{-4}$ (0·5–5·0)	

a—IC50 K^+ divided by IC50 5-HT. b—95% confidence limits. c—no effect. *$P < 0·05$. Each group represents the mean of 6–7 experiments.

ent concentrations of nifedipine (10–100 μM), verapamil (1–10 μM), diltiazem (10–100 μM), cinnarizine (10–100 μM), trifluoperazine (10–100 μM) and W-7 (10–100 μM) were added to the bath. The relaxation effect caused by these compounds in strips previously contracted by Ca^{2+} was compared with that obtained when $CaCl_2$ was omitted from the bath solution (Ballejo et al 1986). When possible the time necessary to produce 50% relaxation ($t_{\frac{1}{2}}$) was determined. Each preparation was exposed to only one concentration of the tested compounds.

Statistical analysis

In each group of experiments the number of preparations reported was obtained from at least two women. The results are presented, when appropriate, as the mean ± s.e.m. The experimental results were analysed by Student's *t*-test. *P* values less than 0·05 were considered to represent significant differences.

Results

5-HT (1–1000 nM) caused a concentration-dependent contractile response in the isolated human umbilical artery (ED50 = 54 nM, 95% confidence limits: 28–67 nM) and maximal contraction of 3·8 ± 0·4 g of tension.

The contractile responses to Ca^{2+}, 5-HT and K^+ were reproducible for several hours when determined at 60 min intervals. Preparations stored for 24 h, at 4°C, did not differ in their responsiveness from those studied immediately after delivery (results not shown).

When the preparations were maintained in Ca^{2+}-free Krebs solution a similar and progressive time-dependent

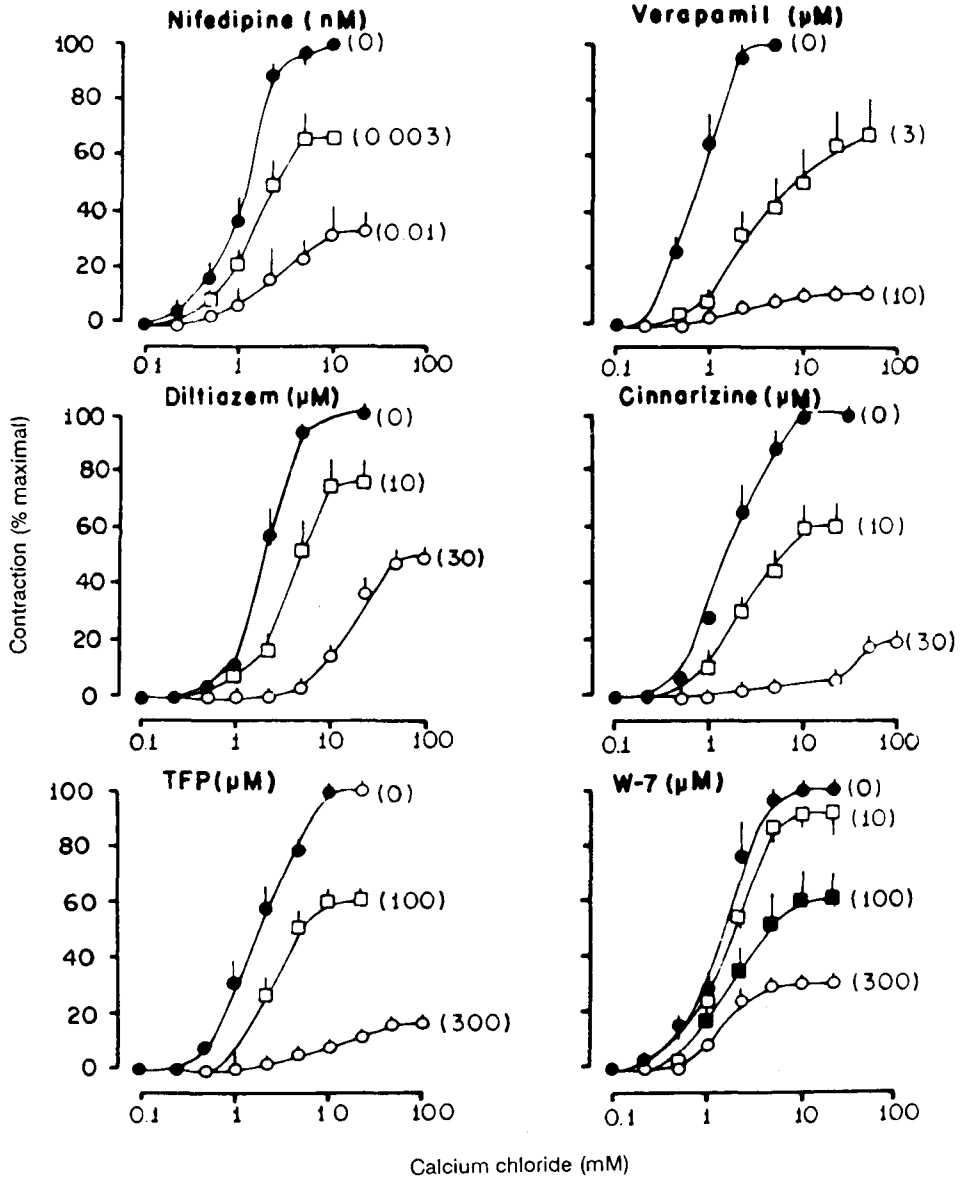

FIG. 3. Mean cumulative concentration-response curves for $CaCl_2$ in human isolated umbilical artery in the absence and in the presence of increasing concentrations of calcium antagonists or calmodulin inhibitors. The concentration of each drug is indicated by the number in parentheses. Each point represents the mean of 5 experiments and the vertical lines the s.e.m.

loss of contractile response to both 5-HT (100 nM) and K^+ (80 mM) was observed ($t_{\frac{1}{2}} \cong 3$ min) (Fig. 1). After 30 min in Ca^{2+}-free solution, both 5-HT and K^+ contractions were reduced to about 90% in relation to the control responses. Addition of Ca^{2+} (2·5 mM) to the medium for 30 min re-established 5-HT and K^+ contractions to about 80–90% of the control response.

Fig. 2 shows the mean cumulative inhibitory concentration response curves obtained for nifedipine (0·1–1000 nM), verapamil (1–1000 nM), diltiazem (0·1–100 μM), cinnarizine (0·3–300 μM), trifluoperazine (0·03–300 μM) and W-7 (1–100 μM) against 5-HT (100 nM) and K^+ (80 mM)-evoked contractions in human isolated umbilical artery. All tested drugs were about 4- to 48-fold more potent in inhibiting 5-HT than K^+-induced contractions when analysed at the IC50 level (Table 1). The rank order of inhibitory potency was: nifedipine > verapamil > trifluoperazine > diltiazem = cinnarizine > W-7. W-7 did not inhibit preparations contracted by K^+. The inhibitory effect of these compounds did not reverse completely even after intermittent washing of the preparations for up to 90 min. As shown in Fig. 3, previous incubation of the Ca^{2+} entry blockers and calmodulin inhibitors in high K^+, Ca^{2+}-free depolarizing solution inhibited in a non-competitive and in a concentration-dependent manner the cumulative contractile responses induced by $CaCl_2$ (0·1–100 mM). As may be observed, nifedipine was about 158 to 7600-fold more potent in inhibiting $CaCl_2$ than 5-HT or K^+-induced contractions, respectively (Table 1). In contrast, verapamil, diltiazem, cinnarizine, trifluoperazine and W-7 were about 6 to 400 and 2- to 39-fold less potent in inhibiting $CaCl_2$ than 5-HT or K^+-induced contractions, respectively (Table 1). The rank order of potency was: nifedipine > verapamil > cinnarizine > diltiazem > trifluoperazine = W-7.

Fig. 4 shows the time-course of the relaxation of sustained $CaCl_2$ (4–7 mM)-induced contractions (ED80) in preparations maintained in K^+-depolarized solution produced by nifedipine (10 and 100 nM), verapamil (1 and 10 μM), diltiazem (10 and 100 μM), cinnarizine (10 and 100 μM), trifluoperazine (10 and 100 μM) and W-7 (10 and 100 μM) or by Ca^{2+} removal. Following Ca^{2+} withdrawal, relaxation occurred rapidly ($t_{\frac{1}{2}} \cong 11$ min). Addition of nifedipine (100 nM), verapamil (10 μM) or diltiazem (100 μM) caused a time-dependent relaxation of sustained $CaCl_2$ contraction with a time-course similar to that produced by Ca^{2+} withdrawal ($t_{\frac{1}{2}} \cong 10$, 9·9 and 17 min, respectively). In contrast, trifluoperazine (100 μM) caused a slow relaxation ($t_{\frac{1}{2}} \cong 30$ min), whereas W-7 caused a slight inhibition of about 20%.

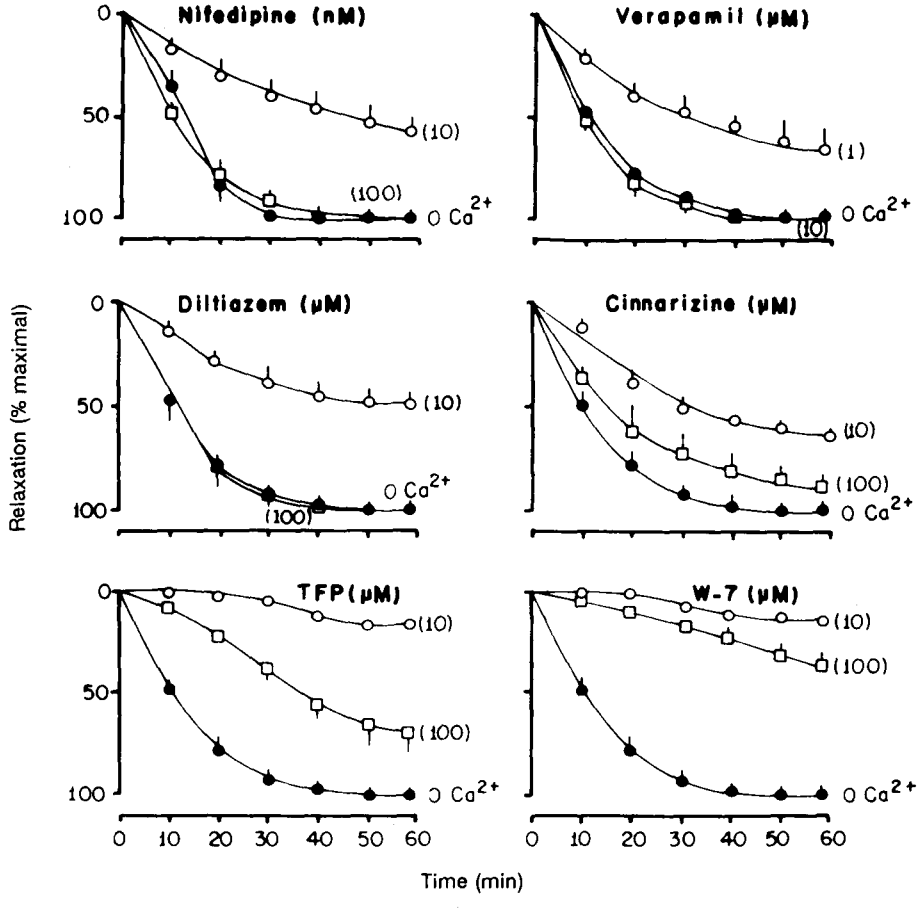

FIG. 4. Time course of the relaxant effects of different concentrations of calcium antagonists and calmodulin inhibitors compared with those produced by omission of $CaCl_2$ (O) from the bath solution on depolarized human umbilical artery. The concentration of these compounds in the bath is indicated by the numbers in parentheses. Each point represents the mean of 7 experiments and the vertical lines the s.e.m.

Discussion

The present study demonstrates that in human umbilical artery both K^+- and 5-HT-induced contractions are dependent on extracellular Ca^{2+} since both contractions were greatly attenuated following Ca^{2+} withdrawal from the nutrient solution ($t_{\frac{1}{2}} \cong 3$ min). In this preparation, the tested drugs acted as typical non-competitive antagonists against $CaCl_2$-induced contractions in depolarizing solution and were significantly more potent in inhibiting 5-HT than K^+-evoked contractions.

Vascular receptors mediating 5-HT contraction in the human isolated umbilical artery (Brown et al 1987) as well as in other vascular beds have been shown to be of the 5-HT_2 subtype. It is suggested that concentrations evoked by 5-HT appear to be primarily the result of an enhanced influx of extracellular Ca^{2+} via voltage or receptor operated Ca^{2+} channels (Van Nueten 1983; Ratz & Flaim 1985; Roth et al 1986). Considering that in human umbilical artery the potencies of Ca^{2+} entry blockers are significantly greater against 5-HT than K^+-evoked contractions, it is possible that in this preparation the activation of 5-HT receptors may be coupled with voltage operated Ca^{2+} channels. Since no electrophysiological studies relating to the action of 5-HT on human umbilical artery are available, this possibility remains to be investigated. Under the same experimental conditions, the putative calmodulin inhibitors, trifluoperazine and W-7, were also significantly more potent against 5-HT- than K^+-evoked contractions, indicating that in this tissue these drugs are acting through different mechanisms compared with those reported in other preparations (Karaki et al 1982; Prozialeck & Weiss 1982; Spedding 1982; Cohen et al 1986).

It is well known that in many smooth muscles different classes of Ca^{2+} entry blockers and some calmodulin inhibitors cause a parallel and concentration-dependent rightward displacement of the concentration-response curve to $CaCl_2$ which is primarily dependent on Ca^{2+} influx (Spedding 1982; Calixto & Loch 1985; Kenakin & Beek 1985). The fact that in human umbilical artery the potencies of Ca^{2+} entry blockers against K^+- and $CaCl_2$-induced contractions are not similar, together with the finding that these compounds exert a typical non-competitive antagonism against $CaCl_2$ contractile effects, indicate that voltage operated Ca^{2+} channels, if present in this preparation, differ greatly from those reported in other tissues (Cavero & Spedding 1983; Loutzenhiser et al 1985; Godfraind et al 1986). These discrepancies also may be partially due to differences in Ca^{2+} sensitivity between tissues in depolarized medium (Spedding 1982; Ballejo et al 1986). Furthermore, compared with nifedipine, the other tested substances were less effective in inhibiting $CaCl_2$ than K^+-contractions. In contrast, trifluoperazine and W-7 were significantly less potent in inhibiting $CaCl_2$-than K^+-evoked contractions but they caused a slow relaxation in Ca^{2+}-precontracted preparations. These results did not permit us to ascertain whether these compounds interfere directly with calmodulin binding sites in human umbilical artery. It is possible that additional sites of action for these drugs may be involved in their relaxant effect in this preparation. Interestingly, nifedipine, verapamil and diltiazem rapidly relaxed Ca^{2+} precontracted preparations, whereas cinnarizine produced a slow relaxation ($t_{\frac{1}{2}} \cong 17$ min). This inhibitory effect may reflect differences in the mechanism of action of these compounds in reducing Ca^{2+} availability by interfering either with Ca^{2+} influx or acting at intracellular sites (Saida & Van Breemen 1983; Spedding 1983; Cohen et al 1986).

In conclusion, the present results indicate that the action of different classes of Ca^{2+} entry blockers and calmodulin inhibitors on human isolated umbilical artery markedly contrast with the pharmacological effect observed for these compounds in other vascular smooth muscles (Muller-Schweinitzer & Neumann 1983; Cauvin et al 1984). Such differences may be related to particular properties of this vascular bed, including lack of innervation, susceptibility to hormonal modulation, short period of life and transport of venous blood.

Acknowledgements

This work was supported from grants from CNPq and FINEP (Brazil). We are grateful to Ms Maria C. A. Neves for technical assistance.

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