Early and late contraction induced by ouabain in human umbilical arteries

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1 Ouabain (3×10^{-7} – 10^{-4} M) evoked a biphasic contraction in human umbilical artery that consisted of an early and a late contraction. The Ca^{2+} -antagonists, verapamil (10^{-7} – 10^{-5} M), diltiazem (10^{-7} – 10^{-5} M) and nifedipine (10^{-9} – 10^{-7} M) inhibited the early but not the late contraction. Caffeine (5 mM) changed neither the magnitude of the peak of the biphasic contraction nor the time needed to reach it.

2 Sodium concentration reduction (140 to 0 mM, replaced by N-methyl-D-glucamine, NMG) produced dose-dependent contraction of the arterial strip in 2.5 mM Ca^{2+} solution after the first treatment with verapamil (10^{-5} M) and caffeine (5 mM), but not in Ca^{2+} -free solution.

3 After prior treatment with verapamil and caffeine, the amplitude of the ouabain (10^{-4} M)-induced late contraction varied, depending on the concentration of Ca^{2+} (0–2.5 mM) in the medium.

4 Amiloride (5×10^{-5} – 5×10^{-4} M), an inhibitor of the Na^{+} – Ca^{2+} exchange system, produced dose-dependent inhibition of the late contraction induced by ouabain (10^{-5} M) after prior treatment with verapamil and caffeine.

5 The time needed to reach the peak tension induced by monensin (5×10^{-7} M) together with ouabain (10^{-6} M) was less than that with ouabain alone, but the magnitude of the peak tension was not changed.

6 These results suggest that the early and late contractions caused by ouabain respectively produce a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and Ca^{2+} entry through sodium-calcium (Na^{+} – Ca^{2+}) exchange.

Keywords: Ouabain; human umbilical artery; early contraction; late contraction; amiloride; monensin; Ca^{2+} -antagonist; Na^{+} reduction; Na^{+} – Ca^{2+} exchange

Introduction

Human umbilical arteries are devoid of sympathetic innervation (von Euler, 1938; Davignon *et al.*, 1965; Somylo *et al.*, 1965; Burton & Godfraind, 1974) and are therefore very useful for the study of the myogenic contraction of arterial smooth muscle (Gopalakrishnan *et al.*, 1985).

We have shown in human umbilical arteries, that a biphasic contraction is evoked by ouabain or a K^{+} -free solution (Sato & Aoki, 1988; 1989). This contraction consists of a transient rapid early contraction and a slow continuous late one. The Ca^{2+} -antagonist, nifedipine, inhibits the early contraction and a Ca^{2+} -free solution inhibits both. Therefore, we suggested that the ouabain-induced early contraction is caused by a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. No inhibition of the early contraction by any other Ca^{2+} -antagonist has been demonstrated, and the mechanism of the late contraction has yet to be demonstrated.

We have now investigated the myogenic mechanisms of the ouabain-induced biphasic contraction after prior treatment with verapamil and caffeine in order to inhibit Ca^{2+} influx and Ca^{2+} release from the sarcoplasmic reticulum. We here discuss the contribution of Na^{+} – Ca^{2+} exchange to the late contraction produced by the reduction of the Na^{+} concentration in the medium.

Methods

Umbilical arteries

Human umbilical arteries were taken from 50 umbilical cords of mothers aged 22 to 33 years at normal full-term uncomplicated deliveries. Mothers who had hypertension, diabetes, Rh factor problems, or other overt diseases or who were on medication such as antihistamines, morphine, and adrenergic

drugs, were not included. At delivery 10 cm segments were cut from the umbilical cords midway between the placenta and the infant and immediately placed in modified Krebs-bicarbonate solution (Krebs solution) at 4°C. After removal of the surrounding tissue, the arteries were cut into 2 by 8 mm helical strips within 12 h of delivery.

Tension development

Strips were mounted vertically between hooks in a water-jacketed muscle bath containing 20 ml of Krebs solution at pH 7.4, then bubbled with a mixture of 95% O_2 and 5% CO_2 . The upper end of each strip was connected to the lever of a force-displacement transducer (TB-612T, Nihon Koden Kogyo Co., Tokyo, Japan) in order to record the tension. Each strip was stretched passively to 200% of its original length. The respective initial and final tensions were 4.4 ± 0.7 (mean $\pm$ s.d.) and 2.1 ± 0.3 g. The strips were allowed to equilibrate in the Krebs solution for 5 h the solution being changed at 15 min intervals. Before the start of the experiments, the solution was replaced with 60 mM K^{+} solution in order to evoke the maximum contraction of 2.2 ± 0.2 g which was considered as the 100% response. The high K^{+} solution was then replaced with the Krebs solution, and the tension relaxed to the basal value. Phenoxybenzamine (Pbz, 2×10^{-6} M) was added to the bath fluid 30 min before inducing contractions in order to inhibit endogenous α -adrenoceptor-mediated effects.

Contractile response to ouabain

In Krebs solution, ouabain (10^{-6} M) induced early and late contractions. The late contraction was evoked only after a 30 min prior treatment with verapamil (10^{-5} M) which inhibited Ca^{2+} influx through the Ca^{2+} channels. The dose-response curves for the ouabain-induced contractions were determined from a single-dose stimulation.

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Figure 1 Recordings of contractions: ouabain (10^{-6} M)-induced contraction (a), the late phase of ouabain (10^{-4} M)-induced contraction in Krebs solution (b) and Ca^{2+} -free solution containing ouabain with 0.2 mM EGTA (c).

Ca^{2+} -antagonists

The umbilical strips were treated with a Ca^{2+} -antagonist for 30 min then contracted with ouabain (10^{-6} M). Concentrations of the Ca^{2+} -antagonists were 10^{-7} to 10^{-5} M for verapamil, 10^{-7} to 10^{-5} M for diltiazem and 10^{-9} to 10^{-7} M for nifedipine.

Contractile response to low Na^{+}

The strips were incubated in a verapamil-caffeine solution for 30 min then contracted by reducing the Na^{+} concentration.

Figure 2 Effects of the Ca^{2+} -antagonists verapamil (10^{-7} – 10^{-5} M) (a), diltiazem (10^{-7} – 10^{-5} M) (b) and nifedipine (10^{-9} – 10^{-7} M) (c) on the early (●) or late (○) phase induced by ouabain (10^{-6} M). Results are expressed as percentages of contractions obtained with a high- K^{+} solution. Points show mean and vertical bars show s.d. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Na^{+} was replaced iso-osmotically with N-methyl-D-glucamine (NMG) and pH was adjusted to 7.4 with HCl. After the strips had been incubated in the verapamil-caffeine solution for 30 min and then immersed in the Ca^{2+} -free EGTA solution for 10 min, they were treated with 14 mM Na^{+} in verapamil Ca^{2+} -free solution.

Ca^{2+}

The effect of extracellular Ca^{2+} on a Ca^{2+} -free solution containing 0.2 mM ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was analysed for 10 min before ouabain-induced contraction. The strips were incubated in a verapamil-caffeine solution for 30 min after which they were immersed in Ca^{2+} -free 0.2 mM EGTA solution for 10 min. The Ca^{2+} -free EGTA solution was replaced with verapamil solution containing CaCl_2 (0–2.5 mM) after which the strips were contracted with ouabain (10^{-4} M).

Amiloride

After a 30 min treatment with verapamil and caffeine in the absence or presence of amiloride (5×10^{-5} – 5×10^{-4} M), the strips were contracted with ouabain (10^{-6} M).

Monensin

The strips were immersed in verapamil-caffeine solution for 30 min after which they were contracted with ouabain (10^{-6} M) in the absence or presence of monensin (5×10^{-7} M). The time needed to reach the peak tension and the magnitude of the maximum tension were measured.

Bath fluid

The composition of the Krebs solution at pH 7.4 (in mM) was: NaCl 115.0, KCl 4.7, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 25.0, KH_2PO_4 1.2 and dextrose 10.0. The 60 mM K^{+} -depolarizing solution was made by equimolar substitution of KCl for NaCl. The Ca^{2+} -free solution was produced by replacing CaCl_2 with equimolar MgCl_2 . Ouabain was dissolved in distilled water on the day of the experiment. A stock solution of Pbz was prepared in saline (0.9% NaCl)-ascorbic acid (0.01%) solution, distilled water and ethanol (99.5%) and was kept at -20°C . Solutions of 10^{-1} M verapamil or diltiazem were made by dissolving each in distilled water. A solution of nifedipine (10^{-3} M) was made in ethanol and kept at -20°C and protected from light. Monensin was dissolved in ethanol and diluted with Krebs solution to give a final ethanol concentration of less than 0.2% (Yang *et al.*, 1986). For use, these solutions were diluted with Krebs solution to the desired concentration.

Drugs

The drugs used were verapamil HCl (Eisei Co., Tokyo, Japan), diltiazem (Tanabe Co., Osaka, Japan), nifedipine (Bayer AG, Tokyo, Japan), caffeine (Sigma Chemical Co., Tokyo, Japan), ouabain octahydrate (Sigma Chemical Co., Tokyo, Japan), phenoxybenzamine (Nakarai Chemical Co., Tokyo, Japan), monensin sodium (Sigma Chemical Co., Tokyo, Japan) and amiloride (Merck & Sharp & Dohme S.p.A., Milano, Italy).

Statistical analysis

Data are expressed as means $\pm$ s.d. Statistical significance was assumed when $P < 0.05$. Student's paired *t* test was used.

Results

Ouabain-induced early and late contraction

Biphasic contraction Ouabain-induced biphasic, dose-dependent contractions at concentration from 3×10^{-7} to

Figure 3 Recordings of 14 mM Na⁺-induced contractions in Krebs solution (a) and in Ca²⁺-free solution containing verapamil and caffeine (b).

10^{-4} M (Sato & Aoki, 1988; 1989). The contraction consisted of a transient rapid early contraction and a slow continuous late one (Figure 1a).

Early contraction in Ca²⁺-antagonist and Ca²⁺-free EGTA solution The early contraction was inhibited by the Ca²⁺-antagonists verapamil, diltiazem and nifedipine (Figures 1b and 2), with complete inhibition of the contraction by Ca²⁺-free 0.2 mM EGTA solution (Figure 1c). The Ca²⁺-antagonists did not inhibit the late contraction (Figures 1b and 2); whereas, the Ca²⁺-free EGTA solution completely inhibited both the early and late contractions (Figure 1c).

Reduction of Na⁺ concentration-induced contractions

The reduction of Na⁺ from 140 to 0 mM in the verapamil solution produced dose-dependent contraction of the strips (Figures 3a and 4a). The 14 mM Na⁺-induced contraction was inhibited by Ca²⁺-free solution after prior treatment with verapamil and caffeine and subsequent treatment with Ca²⁺-free EGTA solution for 10 min (Figure 3b). The relationship between the rate of tension development and Na⁺ is plotted in Figure 4b. The rate when Na⁺ was reduced cannot be

Figure 4 Dose-response curve for Na⁺ reduction after treatment with verapamil together with caffeine (a). The tension development in 0 mM Na⁺ was taken as 100%. Points show mean and vertical bars show s.d. ($n = 5$). Relation between Na⁺ and the relative rate of tension development after treatment with verapamil and caffeine (b). The rate of tension development was calculated from the rising slope, 100% being taken as the development in 0 mM Na⁺. Each symbol represents the mean of data for two to four strips. The absolute rates of tension development in 0 mM Na⁺ were 740.0 ± 42.4 mg min⁻¹ ($n = 3$).

Figure 5 The effect of amiloride on the late phase of ouabain (10^{-6} M)-induced contraction. The results are expressed as a percentage of the contraction obtained with high-K⁺ solution. Five strips were used for each amiloride concentration. Points show mean and vertical lines show s.d. * $P < 0.05$; ** $P < 0.01$.

Figure 6 Recording showing the late phase of ouabain (10^{-5} M)-induced contraction in the presence of verapamil and caffeine (a). The effects of amiloride (10^{-4} M) on the late phase of ouabain (10^{-5} M)-induced contraction (b). Recording (c) shows the late phase of ouabain (10^{-6} M)-induced contraction. The effects of monensin (5×10^{-7} M) on the late phase of ouabain (10^{-6} M)-induced contraction (d).

attributed to Ca^{2+} entry through voltage-sensitive Ca^{2+} channels or to Ca^{2+} release from the sarcoplasmic reticulum because the media contained verapamil and caffeine. Nevertheless, the low Na^+ contractions were entirely dependent upon the external Ca^{2+} .

Effects of Ca^{2+} concentration on the late contraction

After a 30 min treatment with verapamil and caffeine followed by a 10 min treatment with the Ca^{2+} -free EGTA solution, the strips were contracted with ouabain (10^{-4} M) in a solution containing Ca^{2+} up to 2.5 mM. The late contraction was dose-dependent (Figure 1b,c).

Effects of amiloride and monensin on the late contraction

After a 30 min treatment with amiloride (5×10^{-5} M to 5×10^{-4} M), in the presence of both verapamil and caffeine, amiloride inhibited the ouabain (10^{-5} M)-induced contraction dose-dependently (Figures 5 and 6a,b).

Effects of monensin on the late contraction

The time needed to reach the peak tension induced by monensin (5×10^{-7} M) together with ouabain (10^{-6} M) was less than with ouabain alone, but the magnitude of the peak tension was not changed (Figure 6c,d).

Discussion

Ouabain-induced contractions are myogenic or neurogenic depending on the type of artery. A myogenic mechanism for the contraction of arterial smooth muscle has been shown in rat aorta (Karaki *et al.*, 1978) and cat cerebral artery (Marin *et al.*, 1988); whereas a neurogenic mechanism has been demonstrated in dog mesenteric artery and rat tail artery (Bonaccorsi *et al.*, 1977) and in cat femoral artery (Marin *et al.*, 1988). Human umbilical arteries are devoid of sympathetic innervation (Davignon *et al.*, 1965) which suggests that ouabain-induced contractions are myogenic.

Ouabain-induced contractions have been reported to be monophasic (Ozaki *et al.*, 1982; Marin *et al.*, 1988) or biphasic (Hayashi & Park, 1984; Sato & Aoki, 1988; 1989). Ouabain-induced contraction of dog mesenteric arteries was shown to produce a biphasic contraction that consisted of an early and late contraction. The early contraction was neurogenic because of its inhibition by phentolamine, and the late one myogenic because it was not inhibited by phentolamine, atropine, chlorpheniramine, cimetidine, saralasin, aspirin or methysergide (Hayashi & Park, 1984). We earlier reported that in contrast to the contraction of dog mesenteric arteries, the ouabain-induced biphasic contraction of human umbilical arteries is myogenic for both the early and late contractions (Sato & Aoki, 1988; 1989). There is evidence that ouabain-

induced contraction of arterial smooth muscle is evoked by the release of catecholamine from adrenergic nerves (Katsuragi *et al.*, 1977), release of Ca^{2+} from sarcoplasmic reticulum (Lamb *et al.*, 1988); that there is either an increase in Ca^{2+} entry or a decrease in Ca^{2+} extrusion via the Na^+ - Ca^{2+} exchange system (Bohr *et al.*, 1969; Ito *et al.*, 1981; Ozaki *et al.*, 1978; 1982); and that membrane depolarization leads to activation of voltage-sensitive Ca^{2+} channels (Hermsmeyer & Harder, 1986). Our study has demonstrated that in human umbilical arteries, the Ca^{2+} antagonists verapamil, diltiazem and nifedipine inhibit ouabain-induced early contraction but not late contraction and that a Ca^{2+} -free EGTA solution completely inhibits both the early and late contractions. These findings suggest that the ouabain-induced early contraction may depend on a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Prior treatment with verapamil and caffeine inhibited the early ouabain contraction but not the late one. As the effect of this treatment is to inhibit the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels and to release Ca^{2+} from the sarcoplasmic reticulum, the mechanism of late contraction may not be dependent on Ca^{2+} influx through voltage-sensitive Ca^{2+} channels.

Ca^{2+} entry through Na^+ - Ca^{2+} exchange has been reported to evoke the contraction of arterial smooth muscle (Blaustein, 1974; Aicken *et al.*, 1984; Bolton, 1985; Ashida & Blaustein, 1987; Bova *et al.*, 1988). We have demonstrated here that ouabain-induced contraction is dependent on the Ca^{2+} concentration in the bath fluid and that this contraction is inhibited dose-dependently by amiloride.

The time needed to reach the maximum contraction induced by monensin together with ouabain was less than with ouabain alone. The Na^+ reduction-induced contraction was inhibited by a Ca^{2+} -free solution. Taken together, these results suggest that the mechanism for the late contraction probably depends on Ca^{2+} entry through Na^+ - Ca^{2+} exchange in human umbilical arteries.

It has been reported that Na^+ had to be markedly reduced to obtain a significant increase in tension development in guinea-pig aorta (Ozaki *et al.*, 1978); but, in our study on human umbilical arteries half maximal contraction took place with about 90 mM Na^+ . This shows that the artery is very sensitive to changes in Na^+ . The relation between the rate of tension development and Na^+ in human umbilical arteries showed a shift to the right as compared to the relation in rat aorta or bovine tail artery (Ashida & Blaustein, 1987).

In conclusion, in human umbilical arteries ouabain-induced contraction consists of an early and late contraction, the former probably being dependent on a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and the latter on Ca^{2+} entry through Na^+ - Ca^{2+} exchange.

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