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Decreased contraction to phenylephrine by ouabain in 2K-1C rat aorta is modulated by the endothelium

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Abstract

The effects of ouabain were studied on the contraction stimulated with phenylephrine or KCl in intact endothelium and denuded aortic rings isolated from normotensive (2K) and renal hypertensive 2 kidney-1clip (2K-1C) rats. Ouabain did not change the basal tone of aortic rings. Ouabain (1 nmol/l) had no effect on the contraction to phenylephrine in all the artery groups studied. Ouabain (10 nmol/l) decreased the E_{max} to phenylephrine in intact endothelium arteries from 2K-1C. By contrast, ouabain (10 nmol/l) had no effect on the contraction to KCl. Ouabain induced membrane depolarization measured by confocal image with Di-4-ANEPPS dye, that was greater in 2K than in 2K-1C rat aorta smooth muscle cells. In conclusion, ouabain (10 nmol/l) decreased the contractile responses to phenylephrine only in 2K-1C rat aortic rings with intact endothelium. Interestingly, 10 nmol/l ouabain depolarizes the smooth muscle cells but this depolarization level is not enough to alter the phenylephrine or KCl-induced contractions. Our results indicate that the endothelium modulates the vascular action of ouabain.

Keywords: Renal hypertension; Ouabain; Contractile response; Membrane depolarization; Endothelium

1. Introduction

Na-K-ATPase has an important role in the maintenance of the vascular tone and vascular contraction (Blaustein, 1993). In turn, cells become depolarized, internal sodium concentration and internal calcium increases by exchange for sodium via Na-Ca exchanger or via voltage-operated Ca^{2+} channels. This extra calcium is stored, increasing the intracellular pool sensitive to vasoconstrictor agents (Blaustein and Hamlyn, 1983). It has described an endogenous ouabain-like inhibitor of Na-K-ATPase that is found in nanomolar concentrations in the plasma of several animals, including rats (Manunta et al., 1994; Ludens et al., 1992). Evidences emerged suggesting that plasma levels of this inhibitor are increased in some animal models of hypertension, particularly those characterized by volume expansion. Na-K-ATPase inhibition also depolarizes the vascular smooth muscle cell membrane by about 10 mV, and this depolarization has been shown to sensitize blood vessels to contractile agonists such as norepinephrine (Abel et al., 1981; Hendricks and Casteels, 1974). Transmembrane voltage-sensitive fluorescent dyes have been used to monitor fast events such as potential action and membrane polarization produced by electrical stimulation (Fink et al., 1998).

As demonstrated by Rossoni et al. (1999) ouabain in nanomolar concentrations functionally inhibits the Na-K-ATPase and stimulates the release of a K^+ channel activator, which is endothelium-dependent. The endothelium can modulate the actions of ouabain by releasing factors or might alter the synthesis and/or release of a relaxing factor derived from the endothelium (Woolfson and Poston, 1991). However, in the present study we have found that the endothelium modulates the vascular actions of ouabain in renal hypertensive 2 kidney-1 clip rats, but not in normotensive 2K rats. Our results are against the hypothesis that ouabain can increase vascular resistance and induce hypertension in hypertensive models with volume expansion.

Therefore, the aim of the present study was to evaluate the effect of ouabain in nanomolar concentrations on the contractile responses induced by phenylephrine or KCl, in intact

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endothelium and denuded aortic rings isolated from 2K and 2K-1C rats. Our results indicate that the endothelium modulates the vascular action of ouabain. Although ouabain per se does not induce vascular tone changes, we measured the smooth muscle cells membrane potential in order to evaluate the contribution of smooth muscle cells membrane depolarization to the effect of ouabain.

2. Materials and methods

2.1. Animals

Experimental protocols followed standard policies of the University of São Paulo's Animal Care and Use Committee. Renovascular hypertension was induced in rats following the 2K-1C Goldblatt model. Briefly, male Wistar rats (180 to 200 g) were anesthetized, and after a midline laparotomy, a silver clip with 0.20 mm internal diameter was placed around the left renal artery. The 2K rats were submitted to laparotomy only.

2.2. Arterial pressure measurements

Six weeks after surgery, the systolic arterial pressure was measured by the tail-cuff method. Rats were considered to be hypertensive when systolic pressure was >160 mm Hg.

2.3. Preparation of rat aortic rings

Following the arterial pressure recordings, the rats were killed by decapitation under anesthesia, and the thoracic aortas were isolated. Aortic rings, 4 mm in length, were placed in bath chambers (10 ml) for isolated organs containing modified Krebs salt solution at 37 °C, pH 7.4, continuously bubbled with 95% O_2 and 5% CO_2. Two fine stainless steel holders were placed through the lumen of the aortic rings, one of the holders was fixed to the tissue chamber and the other was connected to a F-60 force-displacement transducer and the responses were recorded on a physiograph. The aortic rings were submitted to a tension of 1.5 g during the 60 min equilibration period before the addition of a given drug. At the beginning of the experiments, the aortas were stimulated for three times with 100 nmol/l norepinephrine to test their functional integrity by the reproducible responses. In some experiments, the endothelium was removed by gently rubbing the luminal surface of the vessel and endothelium removal was confirmed by the lack of relaxation in response to 1 μmol/l acetylcholine in aorta rings pre-contracted with 100 nmol/l norepinephrine. Artery rings were considered to have an intact functional endothelium, when acetylcholine (1 μmol/l) produced a relaxation of more than 80%.

2.4. Concentration-response curves

After the equilibration period, the aortic rings were stimulated with increasing concentrations of ouabain (0.1 nmol/l to 0.5 μmol/l). Another group of preparations were stimulated with increasing concentrations of phenylephrine (0.1 nmol/l to 10 μmol/l), cumulatively added to the bath before and after the incubation with ouabain (1 and 10 nmol/l). Increasing concentrations of KCl (4.7 mmol/l to 120 mmol/l) were also cumulatively added to the bath before and after the incubation with ouabain (10 nmol/l).

2.5. Cells isolation

The smooth muscle cells were isolated from 2K and 2K-1C rat aortas and submitted to an enzymatic digestion by collagenase. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 20 mmol/l HEPES (pH 7.4), 10,000 U/ml penicillin, and 10,000 μg/ml streptomycin and plated onto glass coverslips. After 6 h of incubation at 37 °C in a humidified atmosphere of 5% CO_2 the cells adhered to the plastic or to the coverslip. The medium was aspirated to remove nonattached smooth muscle cells. The medium was changed every 2 days. After 7–9 days, the culture had formed confluent monolayers either in plastic bottle (spectro-fluorimeter) or in coverslip (confocal microscopy). Smooth muscle cells cultured in plastic bottle, in log phase were removed from the stock suspension and washed in Hanks' solution, detached with trypsin, and resuspended in Hanks' solution in order to be used in suspension. Viability of the cells at this stage was determined by exclusion of 0.4% trypan blue dye, invariably greater than 95%.

2.6. Confocal microscopy

Fluorescence emission and the images were recorded using a laser scanning confocal microscope (LEICA TCSNT) coupled to a LEICA DMIRBE inverted microscope equipped with a 63× objective. For Di-4-ANEPPS detection, the wavelength excitation was 488 nm from krypton/argon laser. Fluorescence emission was detected using a BP530/30 set filter. The time calibration for loading the cells was performed for 20 min. Between 5 and 20 min, no changes in the maximum intensity of fluorescence was detected. Then, the time for loading was established as 5 min.

2.7. Solutions and drugs

Composition of Krebs salt solution was the following (in mmol/l): NaCl 130, $CaCl_2$ 1.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 4.7, $NaHCO_3$ 14.9, dextrose 5.5 and EDTA 0.03. In experiments with high $[K^+]_o$, Krebs salt solution was prepared by equimolar replacement of NaCl with KCl. Phenylephrine, acetylcholine, norepinephrine, and ouabain were dissolved in deionized water and incubated in Krebs salt solution. Acetylcholine, ouabain, norepinephrine and phenylephrine were obtained from Sigma-Aldrich (St. Louis, MO). The potential-sensitive probe, Pyridinium 4-(2-(6-(dibutylamino)-2-naphtha-lenyl) ethenyl)-1-(3-sulfopropyl)-, hydroxide, inner salt (Di-4-ANEPPS) was obtained from Molecular Probes (Eugene, OR).

2.8. Data analysis

Results are expressed as mean±S.E.M. and n indicates the number of animals. The concentration of the contractile agents producing a half-maximal response (EC_{50}) was determined after logit transformation of the normalized concentration-response curves and is reported as the negative logarithm (−log EC_{50}=pD_2 values) of the mean of the individual values of each tissue by the use of the Prism GraphPad 3.02 software (GraphPad Software Inc., San Diego, CA). The maximum effect was considered as the maximal amplitude response reached in concentration-effect curves for contractile agents. For statistical analysis, multiple comparisons were made by one-way variance followed by the Dunnett post test for all pairwise comparisons. The level of significance was set at $P<0.05$.

3. Results

3.1. Study 1: general aspect

Systolic arterial pressure was significantly higher in 2K-1C rats (180±10 mmHg, $n=12$) than in 2K rats (120±10 mm Hg, $n=15$).

3.2. Study 2: effect of ouabain on the aortic rings precontracted with phenylephrine

The addition of the Na-K-ATPase inhibitor ouabain had no effects on the resting vascular tone in both 2K and 2K-1C rat aortic rings. In intact endothelium 2K and 2K-1C rat aortic rings, ouabain (1 nmol/l) had no effect on the contractile response induced by phenylephrine. The values obtained are represented on Table 1. In the same way, in denuded aortic rings from 2K and 2K-1C rats no differences were observed before and after ouabain (1 nmol/l). Comparing the results obtained in 2K and 2K-1C rat aortic rings, we observed that the contractile response stimulated with phenylephrine was lower in 2K-1C rats (E_{max} 1.26±0.11 g, $n=7$) than in 2K rats (E_{max} 2.82±0.34 g, $n=5$, $P<0.01$) denuded aortic rings.

As shown in Fig. 1A, in intact endothelium aortic rings, ouabain (10 nmol/l) reduced the E_{max} only in 2K-1C rats aortic rings (from: 1.58±0.13, $n=6$ to 1.19±0.12 g, $n=6$, $P<0.01$), but pD_2 values were not altered. In 2K rat aortic rings ouabain had no effect on phenylephrine-induced contractile response. In denuded arteries, ouabain had no effects in both 2K and 2K-1C rat aortic rings (Fig. 1B).

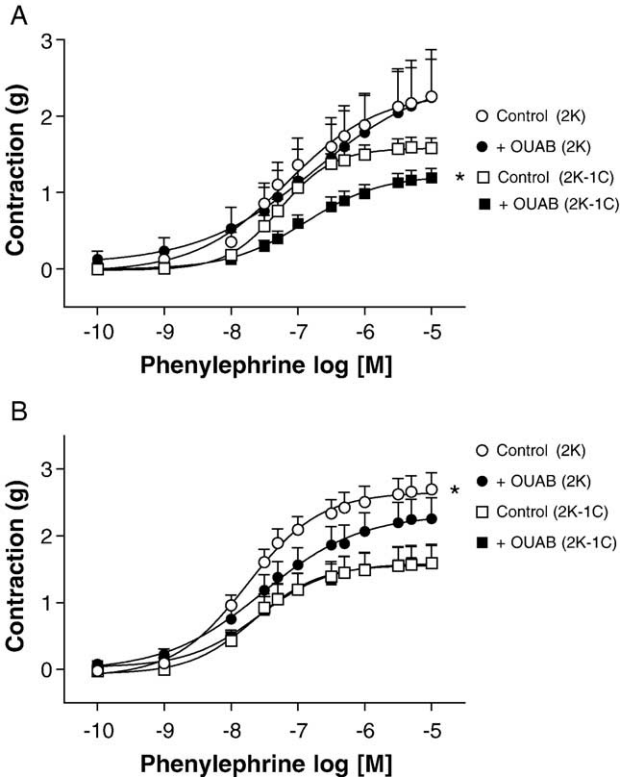

Fig. 1. Effects of 10 nmol/l ouabain (OUAB) on phenylephrine-induced contractile responses in aortic rings from 2K and 2K-1C rats with intact endothelium (A) and denuded arteries (B). Values are means±S.E.M. and are represented as g of contraction. Differences in the maximum contraction to phenylephrine is indicated as *, $P<0.01$, 2K-1C rat aortas versus 2K rat aortas; #, $P<0.05$, presence versus absence of ouabain in 2K-1C rat aortas.

In the absence of ouabain, the pD_2 value was greater in denuded 2K rat aortic rings (7.77±0.14, $n=10$) than in intact endothelium arteries (7.15±0.14, $n=5$, $p<0.05$), as shown on Table 1. On the other hand, endothelium removal had no effect on the E_{max} (2.70±0.25 g and 2.26±0.49 g). In the same way, in 2K-1C arteries, the E_{max} was similar in denuded arteries (1.59±0.28 g, $n=6$) and in intact endothelium (1.58±0.13, $n=6$, $P<0.05$) aortic rings, and the pD_2 values were not altered (7.54 ±0.16 and 7.30±0.14). The E_{max} in denuded arteries was greater in 2K rats (2.70±0.25 g, $n=10$) than in 2K-1C rats (1.59±0.28 g, $n=6$, $P<0.05$).

Table 1
pD_2 values and maximum contractile effect (E_{max}) of phenylephrine in 2K and 2K-1C rat aorta with endothelium or denuded before and after ouabain

Drug	pD_2 (n)				Emax (g tension)			
	Endothelium		Denuded		Endothelium		Denuded	
	2K	2K-1C	2K	2K-1C	2K	2K-1C	2K	2K-1C
Control	7.44±0.22 (8)	7.36±0.08 (5)	7.81±0.48 (5)	7.34±0.18 (7)	2.40±0.38	1.41±0.28	2.82±0.34	1.26±0.11[a]
Ouabain (1 nM)	6.95±0.1 (8)	7.04±0.08 (5)	7.57±0.27 (5)	7.43±0.17 (7)	2.19±0.34	1.14±0.17	2.52±0.18	1.27±0.14
Control	7.15±0.14 (5)	7.30±0.14 (6)	7.77±0.14 (10)[b]	7.54±0.16 (6)	2.26±0.49	1.58±0.13	2.70±0.25	1.59±0.28[c]
Ouabain (10 nM)	6.69±0.21 (5)	6.91±0.23 (6)	7.53±0.17 (10)	7.47±0.14 (6)	2.24±0.63	1.19±0.12[b]	2.26±0.31	1.60±0.26

[a] Denotes significant difference between 2K and 2K-1C denuded preparations ($p<0.01$).
[b] Denotes significant difference between control and ouabain 2K-1C preparations ($p<0.01$).
[c] Denotes significant difference between 2K and 2K-1C denuded preparations ($p<0.05$).

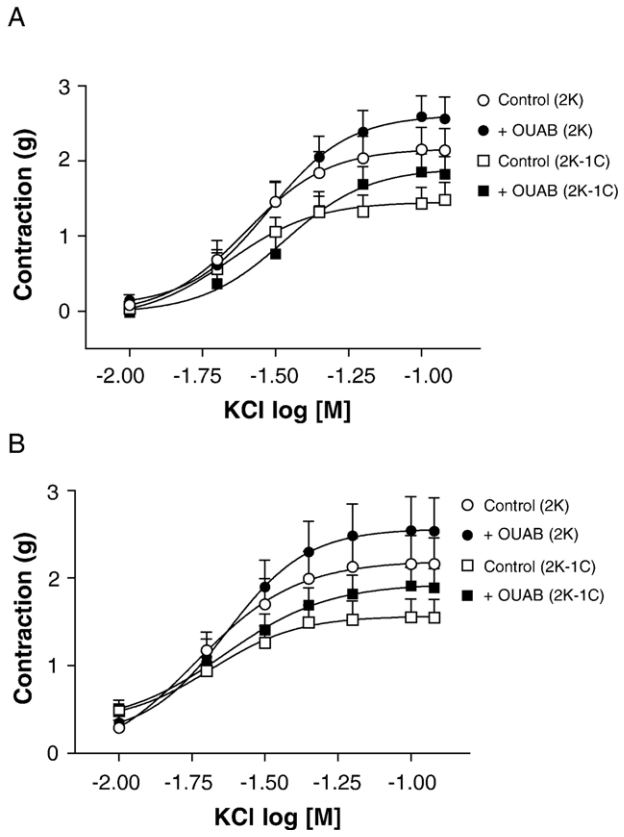

Fig. 2. Effects of 10 nmol/l ouabain (OUAB) on KCl-induced contractile responses in aortic rings from 2K and 2K-1C rats with intact endothelium (A) and denuded arteries (B). Values are means ± S.E.M. and are represented as g of contraction.

3.3. Study 3: effect of ouabain on the aortic rings precontracted with KCl

As shown in Fig. 2, the contractile responses stimulated with increasing concentrations of KCl in intact endothelium and in denuded aortic rings were concentration-dependent in 2K and 2K-1C rats, in the absence or in the presence of ouabain (10 nmol/l). Endothelium removal or ouabain incubation had no effects on the contractile responses induced by KCl (Fig. 2).

3.4. Study 4: effect of ouabain on membrane depolarization and measurement of membrane potential in aorta smooth muscle cells

The cell membrane depolarization was caused by externally added 10 nmol/l ouabain. The internalization of the dye Di-4-ANEPPS was obtained within 20 min. The fluorescence intensity increased in the presence of ouabain (Fig. 3). The effect of ouabain in depolarizing the cell membranes was higher in 2K than in 2K-1C rats aorta smooth muscle cells ($P<0.05$). In the cells incubated with ouabain (10 nmol/l) and Di-4-ANEPPS, the fluorescence intensity was increased in 17.48 ± 0.27% ($n=4$) for 2K rat cells and in 12.71 ± 0.65% for 2K-1C rat cells ($n=4$). The resting membrane potential of 2K-1C rat aorta cells membrane was less negative (−55 mV, $n=6$) than that of 2K rats aorta cell membrane (−65 mV, $n=10$).

4. Discussion

With the present study we demonstrated that in spite of the fact that ouabain does not produce any contractile response nor alteration of the resting vascular tone in aortic rings with and without endothelium, it depolarizes the smooth muscle cells membrane of 2K and 2K-1C rats aorta. As we have reported before, 2K-1C rat aorta cells membrane is more depolarized than 2K cells (Callera et al., 2000; Sguilla et al., 2003). These results are in accordance to other studies showing that ouabain in nanomolar concentrations can depolarize the membrane of vascular smooth muscle cells. In addition, the endogenous factor “ouabain like” present in similar concentrations in the plasma could inactivate the enzyme Na-K-ATPase, leading to the increase of intracellular Na^+ concentration (Vassallo et al., 1997). In the present study, we show that the depolarization induced by ouabain seems not be enough to induce contractions.

Interestingly, we have shown that in the absence of ouabain in denuded arteries, the maximum contractile response induced by phenylephrine was greater in 2K than in 2K-1C rat aortic rings. In the present study, ouabain (1 nmol/l) had no effect in normotensive 2K rats and renal hypertensive 2K-1C rat aortic

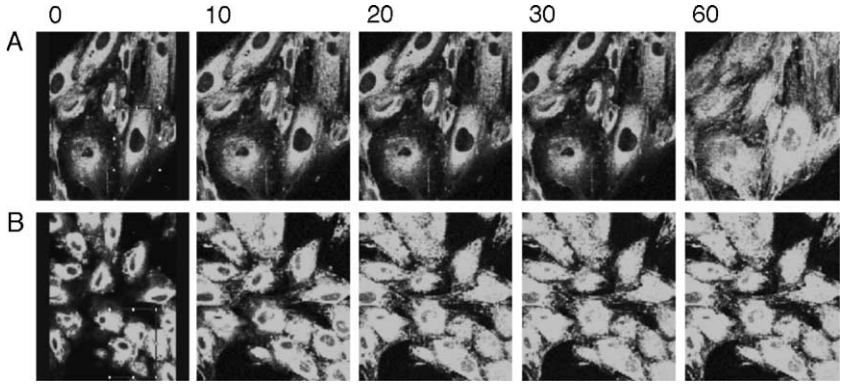

Fig. 3. Effect of ouabain on membrane potential of aorta smooth muscle cells. Cells were loaded with Di-4-ANEPPS (1 μmol/l) and when a stable signal was obtained, the solution was replaced by the medium containing 10 nmol/l ouabain. The images of changes in membrane potential were collected immediately before the medium change in 2K rat aorta (A) and 2K-1C rat aorta (B) cells. Membrane depolarization was measured at 0 (in absence of ouabain) and in 10, 20, 30 and 60 s after addition of ouabain, as indicated.

rings with intact endothelium and in denuded aortic rings. Ouabain (10 nmol/l) reduced the contractile response induced by phenylephrine only in intact endothelium aortic rings of 2K-1C rats. As suggested by Rossoni et al. (1999), ouabain in the concentration of 10 nmol/l, could release relaxing endothelium factors that relaxes the tail arteries of normotensive rats. Probably, this factor is the endothelium-derived relaxing factor (EDHF) that could be activating the Na^+-K^+-ATPase through membrane hyperpolarization. Therefore, ouabain would reduce the contractile responses stimulated with agonists that mobilize Ca^{2+} such as phenylephrine. The authors reported that in addition to EDHF, nitric oxide also participates of the reduction of the contractile response to phenylephrine (Rossoni et al., 2002). In contrast, Vassallo et al. (1997) have showed that the pretreatment with ouabain increased the contractile response to phenylephrine in normotensive and hypertensive (SHR) in vivo and in vitro. In the same way, Rossoni et al. (2001) have demonstrated the effect of ouabain in the models of hypertension 1K-1C rats and Doca-salt rats. According to Doris (1996) the endothelium negatively modulates the vasoconstriction elicited by Na-K-ATPase inhibition by the release of nitric oxide (NO) or endogenous digitalis-like factor. Moreover, as reported by Chen et al. (2003) the abnormal activation of Na-K-ATPase is associated with an overproduction of NO-cGMP.

The greater contractile response of 2K rat denuded aortic rings suggest that relaxing endothelium-derived factors, such as NO, could be contributing to the reduction of phenylephrine-induced contractile response. According to Nava et al. (2000), acute hypertension produced by infusion of catecholamines or angiotensin II can induce NO release. It can be detected in the plasma by the increased concentrations of nitrite. These authors suggest that the endothelial NO-synthase can be auto-regulated in response to acute changes in blood pressure.

It is well known that cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) elicit the contractile response of the aorta smooth muscle cells. Then, the 2K-1C rat aortic rings should present a greater contraction. Otherwise, we have previously reported that the increased basal $[Ca^{2+}]_c$ of 2K-1C rat aorta cells does not contribute to the increased contractile responses induced by phenylephrine. In the same way, in the present study the contractile response to phenylephrine was lower in 2K-1C rats aorta, in spite of the increased $[Ca^{2+}]_c$ measured with Fura-2 (Callera et al., 2001).

Activation of Na-K-ATPase induces membrane hyperpolarization of the vascular smooth muscle cells (Chen et al., 1989). In accordance to these authors, membrane hyperpolarization of the coronary artery is dependent on the activation of Na-K-ATPase and not to the increased conductance to ions K^+. The relaxation induced by acetylcholine that involves membrane hyperpolarization, is not inhibited by ouabain in aorta from 2K-1C rats (Callera et al., 2004). In the present study, ouabain had no effects on the contractile response stimulated with KCl. The membrane depolarization induced by ouabain depends on the increased concentration of ions Na^+in the cells cytosol. Activation of Na-K-ATPase promotes Na^+ efflux and K^+influx, but our data demonstrate that when this transporter is inhibited, the contractile response to KCl is not altered.

Taken together, our results strongly suggest that ouabain reduces contractile response to phenylephrine in 2K-1C rat aortas, which is endothelium-dependent. Therefore, this study demonstrates that depolarization induced by 10 nmol/l ouabain may be not enough to change the contractile responses induced by phenylephrine or KCl. Our data confirm that the endothelium modulates the vascular actions of ouabain, but the results are against the hypothesis that ouabain can increase vascular resistance and induce hypertension in 2 kidney-1 clip renal hypertensive rats.

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