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Study of the mechanisms regulating human umbilical artery contractility

António José Santos-Silva[1], Elisa Cairrão[1,2], Ignacio Verde[3]

[1]Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Avenida Infante Dom Henrique, Covilhã, Portugal
[2]Centro Hospitalar da Cova da Beira, Quinta do Alvito, Covilhã, Portugal
[3]Centro de Investigação em Ciências da Saúde, Universidade daBeira Interior, Avenida Infante Dom Henrique, Covilhã, Portugal; iverde@fcsaude.ubi.pt

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ABSTRACT

We studied the involvement of different types of Ca^{2+} channels, cyclic nucleotides and different kinases in the regulation of human umbilical artery (HUA) contractility. The elucidation of the precise mechanisms regulating the contractility of this artery could be very important to reveal potential therapeutic targets to treat HUA disorders such as preeclampsia. The relevancy of different types of Ca^{2+} channels on the regulation of HUA tonus was analyzed. Among the different Ca^{2+} channel inhibitors used, only the L-type calcium channels (LTCC) inhibition induced relaxation of HUA in Ca^{2+} containing medium. The inhibition of T-type calcium channels (TTCC) or TRP channels did not significantly affect HUA contractility. In presence of Ca^{2+}, the intracellular increase of a cyclic nucleotide (cAMP or cGMP) induces relaxation of HUA, which was almost complete in histamine-contracted HUA, and lower effect was observed in arteries contracted by KCl and serotonin (5-HT). Inhibition of PKA and PKG weakly reduced the relaxations induced by the increase of cAMP and cGMP respectively, suggesting that the relaxation induced by these nucleotides is not totally mediated by the activation of their respective kinases and that other mechanisms are involved. In calcium containing solution, PP2A inhibition produces relaxation of contracted HUA. In KCl contracted arteries, the OA and nifedipine relaxant effects are similar and not additive, suggesting that PP2A could activate LTCC. Besides, the increase of cyclic nucleotides significantly increased the OA effect, suggesting that the effect of PP2A inhibition is independent of the cyclic nucleotide pathways. The contractions induced by KCl, histamine and 5-HT in presence of Ca^{2+} were not significantly affected by ROCK, ERK1/2 or p38MAPK inhibitors. In absence of extracellular Ca^{2+}, histamine and 5-HT elicited contractions of HUA characterized by two components, a rapid phasic contractile component followed by a decrease of the contraction until a tonic component. However, KCl elicited sustained contractions of HUA in absence of extracellular Ca^{2+}. As in presence of calcium, the ERK1/2 and p38MAPK inhibitors did not influence the contractions induced by KCl, histamine or 5-HT in absence of extracellular Ca^{2+}. However, in these conditions, ROCK inhibition significantly relaxed the contractions induced by KCl and reduced the phasic and tonic components of the contraction elicited either by histamine or 5-HT. Our results show that calcium-dependent contractions of HUA depend on Ca^{2+} entry by LTCC, and these channels seems to be positive regulated by PP2A. Cyclic nucleotides mediate HUA vasodilatation but their dependent kinases are not the unique responsible of this effect. HUA is able to contract independently of Ca^{2+} influx by activating the ROCK pathway and/or due to intracellular Ca^{2+} release.

Keywords: Umbilical Artery; Smooth Muscle; L-type Ca^{2+} Channels; ROCK; Cyclic AMP; Cyclic GMP

1. INTRODUCTION

The mechanisms regulating smooth muscle contractility in human umbilical artery (HUA) are very important for optimum gas and nutrient exchange between foetus and placenta. Since the umbilical blood vessels are not innervated, the control of umbilical blood flow depends of vasoactive substances either released locally or existing in the circulation [1,2]. In general, vascular smooth

muscle (VSM) contractions are initiated by receptor or ion channel activation and involve Ca^{2+} dependent and/or independent mechanisms [3]. The increase of cytosolic free Ca^{2+} can be originated by a transient and rapid increase due to the Ca^{2+} release from the sarcoplasmic reticulum and/or by a sustained extracellular Ca^{2+} influx through Ca^{2+} channels [4,5]. This Ca^{2+} increase leads to myosin light chain kinase activation and contraction due to interaction between the thick and the thin myofilaments [6]. VSM relaxation can be mediated by activation of myosin light chain phosphatase which has opposite effects than myosin light kinase activation.

Different types of Ca^{2+} channels have been involved in the control of VSM contractility, such as L-type Ca^{2+} channels (LTCC) [7,8], T-type Ca^{2+} channels (TTCC) [9], store-operated Ca^{2+} channels (SOCC) [10] or stretch-activated channels (SAC) [11,12]. Recently, the SOCC and SAC have been identified as being transient receptor potential (TRP) channels [13]. In VSM, different TRP channels were linked with Ca^{2+} store depletion, G-protein-coupled receptor activation, membrane stretch, pho- spholipid signals and other factors. However, the role of these channels was not clarified yet, probably due to the lack of specific pharmacological tools, such as specific activators and/or inhibitors [14]. The TTCC were mainly involved in the regulation of cell proliferation [15]. Although these channels were involved in smooth muscle contraction by some authors [16], the information linking TTCC with VSM contractility is scant and their relative importance in this process needs further analysis. On the other hand, LTCC seems to be dominant in the regulation of VSM contractility because they have been appointed as the main pathway for Ca^{2+} entry. In HUA, some authors identified different LTCC and TTCC [17] and the LTCC blockers have appeared to be the most potent relaxants of this artery [18]. On the other hand, in smooth muscle cells, LTCC could be regulated by PP2A, although the number of studies is very low and the direction of this modulation has remained somewhat controversial. Some author have shown that PP2A does not modulate LTCC in tracheal smooth muscle [19], but in intestinal smooth muscle a dual effect of phosphatase inhibitors on LTCC has been reported [20]. In human umbilical vein, Groschner *et al*. have suggested that inhibition of PP2A activates LTCC [21].

Concerning the Ca^{2+} independent mechanism, it could involve Ca^{2+}-sensitization mediated by the activation of the RhoA-kinase (ROCK) which causes inhibition of myosin light chain phosphatase, leading to an increase of myosin light chain phosphorylation [5]. In this sense, some authors have reported that rhoA/ROCK pathway can contribute to agonist-induced contractions of HUA. However, this effect seems to be limited to intracellular Ca^{2+}-induced contractions and may be more important in sustaining contractions rather than the initial phase of force development [22]. Other authors have suggested the existence, in VSM cells, of a Ca^{2+}-dependent Rho stimulation mechanism linked to different receptor coupled to G proteins [23]. Also, it has been described that rabbit artery smooth muscle depolarization increases Ca^{2+} sensitization due to ROCK activation [24]. On the other hand, a role of some components of the MAPK cascade and its substrates (ERK1 and ERK2) in modulating the VSM contractility has been also suggested [25]. Some roles of these kinases in the regulation of cell proliferation and differentiation has been established [26] but their role in VSM contraction and relaxation is almost unknown. Some agonists inducing VSM contraction can also activate ERKs [27,28]. In cerebral arteries, it was suggested that ERKs modulate Ca^{2+} sensitivity and contractility [25]. Other authors have observed that 5-HT induces rat aorta contractions involving p38 MAPK or Erk MAPK pathways [29-31].

The cyclic nucleotides, cAMP or cGMP, are the main second messenger involved in the regulation of vasodilation [32]. Intracellular accumulation of cAMP and cGMP can be achieved by stimulation of adenylate or guanylate cyclase, respectively, or by inhibition of phosphodiesterases (PDE) [33]. Concerning cAMP, intracellular increase of this nucleotide induces relaxation of different human arteries [34-36], including HUA [37]. Among the four families of PDE expressed in this smooth muscle (PDE1, PDE3, PDE4 and PDE5), PDE4 has been shown as the key enzyme involved in the regulation of HUA relaxation associated to cAMP [37]. Concerning cGMP, the increase of the intracellular level of this nucleotide also induces artery vasodilatation [38], including HUA vasodilatation [37,39]. PDE5 has been shown as the key enzyme involved in the regulation of HUA relaxation associated to cGMP [37]. Also, distinct authors have described that in different arteries [40,41], including HUA [39], the vasodilatation induced by cGMP is mediated by activation of potassium channels. Increases in cAMP and cGMP activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively [42]. In VSM cells, the inhibition of LTCC by PKG has been reported [43]. However, the cAMP pathway has been suggested to inhibit, to enhance, or to have no effect on smooth muscle LTCC [43]. Also, some authors have suggested that relaxation induced by cyclic nucleotides is not totally mediated by the activation of their respective kinases. In this sense, other mechanisms were involved, such as cross-activation between cyclic nucleotide dependent kinases [44] or the regulation of other proteins having Epac (exchange protein directly activated by cAMP) which activates the small GTPbinding protein Rap1 [45].

Despite of the great importance of HUA before and during childbirth, the mechanisms involved in the regu-

lation of the contractility of this artery have been weakly studied. Devoid of enervation, the regulation of vascular tone of the HUA depends only by the local release of humoral factors, such as 5-HT and histamine [1,2]. In this work we have evaluated the relevancy of some mechanisms involved in the contraction and relaxation of this artery, such as extracellular Ca^{2+}, cyclic nucleotides, Ca^{2+} channels and different kinase types.

2. METHODS

2.1. Tissue Preparation

Umbilical cord pieces of 3-7 cm were obtained from normal term pregnancies with the consent of the donor mothers. All procedures carried out with these samples have been approved by the Ethics Committee of "Centro Hospitalar da Cova da Beira EPE". The umbilical cord samples were collected in sterile physiological saline solution (composition, mM: NaCl 110; $CaCl_2$ 0.15; KCl 5; $MgCl_2$ 2; HEPES 10; $NaHCO_3$ 10; KH_2PO_4 0.5; NaH_2PO_4 0.5; Glucose 10; EDTA 0.49). In order to avoid contamination and tissue degradation, penicillin (5 U/ml), streptomycin (5 μg/ml), amphotericin B (12.5 ng/ml) and antiproteases (leupeptine, 0.45 mg/l; benzamidine, 26 mg/l; and trypsin inhibitor, 10 mg/l) were added to the physiological saline solution. Umbilical artery rings of 3-5 mm were isolated from the surrounding connective tissue. Vascular endothelium was mechanically removed by gentle rubbing with a cotton bud introduced through the arterial lumen. These denuded HUA rings were used to perform contractility experiments.

2.2. Artery Tension Recording

2.2.1. Relaxation Studies in Ca^{2+} Containing Medium

The HUA rings were placed in organ bath chambers (LE01.004, Letica) containing Krebs-bicarbonate solution (composition, mM: NaCl 119, KCl 5.0, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgSO_4$ 1.2, EDTA 0.03, glucose 11) at 37ºC and continuously gassed with carbogen. The artery rings were suspended between two parallel stain- less steel wires and tension measurement was performed using isometric transducers (TRI201, Panlab SA, Spain), amplifier (ML118/D Quad Bridge, ADInstruments), interface PowerLab/4SP (ML750, ADInstruments) and a computerized system with Chart5 PowerLab software (ADInstruments). For analysis, the isometric tension measured has been expressed in milligrams (mg) of force elicited by the artery in presence of drugs. To analyze the relaxation data, we used the percentage of reduction on the maximal contraction induced by the contractile agents. During the resting periods, the organ bath solution was changed every 15 min. Initially, the rings were equilibrated for 60 min until a resting tension of 1000 mg was achieved. After this, the rings were challenged with 5-HT (1μM) to test their viability. Rings that induced a maximal contraction lower than 1 g when challenged with 5-HT were excluded from the study. Afterwards, the rings were contracted using KCl (60 mM), histamine (10 μM) and 5-HT (1 μM). To determine the involvement of distinct types of Ca^{2+} channels, the LTCC blocker nifedipine (10 μM), the TTCC blocker mibefradil (10 μM) and the TRP blocker 2-aminoethoxydiphenyl borate (APB; 100 μM) have been used.

To analyze the involvement of the cAMP or cGMP pathways the following drugs were used in some cases: rolipram (1 μM), a PDE4 selective inhibitor; forskolin (10 μM), an adenylate cyclase activator; KT-5720 (KTa; 1 μM), a PKA inhibitor; sodium nitroprusside (SNP; 10 μM) a guanylate cyclase stimulator; dipyridamol (3 μM), a PDE5 inhibitor; and KT-5823 (KTg; 1 μM), a PKG inhibitor. To evaluate the possible involvement of PP2A, okadaic acid (OA; 5 nM) has been used in some experiments. Control experiments with ethanol, the vehicle used to dissolve some drugs, were always performed.

2.2.2. Relaxation Studies in Ca^{2+}-free Medium

To analyze the HUA contractility in absence of Ca^{2+}, we used a Krebs solution without Ca^{2+} (composition, mM: NaCl 119, KCl 5.0, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, EDTA 0.03, EGTA 0.5, glucose 11). The rings were also contracted using KCl (60 mM), histamine (10 μM) or 5-HT (1 μM).

To analyze the modulation of contractility by some kinases in Ca^{2+}-free contractions, the following drugs were used in some experiments: Y-27632 (Y27; 10 μM), a ROCK inhibitor; PD-98059 (PD; 50 μM) an ERK1/2 inhibitor; and SB-203580 (SB; 25 μM), a p38MAPK inhibitor. In some experiments, nifedipine (10 μM) and OA were also used.

2.3. Drugs and Chemicals

All drugs and chemicals have been purchased from Sigma-Aldrich Quimica (Sintra, Portugal), except forskolin and rolipram, which were purchased from Biogen Cientifica (Madrid, Spain). Forskolin, rolipram and dipyridamol, were initially dissolved in ethanol and all the other drugs were initially dissolved in distilled water. Final solutions were obtained by dilution with Krebs solution. The final concentration of ethanol in the organ bath did never exceed 0.1%.

2.4. Statistical Analysis

Statistical analysis of the data has been performed using the SigmaStat Statistical Analysis System, version 1.00 (1992). Results have been expressed as mean ± s.e.m. of

n experiments. Comparison among multiple groups was analyzed by using a one-way ANOVA followed by Tukey or Dunnet´s post hoc tests to determine significant differences among the means. Comparison among two groups was analyzed by using Student-t test. Probability levels lower than 5% were considered significant ($P < 0.05$)

3. RESULTS

3.1. Effect of Ca^{2+} Channel Inhibitors in Contracted HUA

The HUA rings without endothelium were contracted by KCl (60 mM), histamine and 5-HT. The presence of KCl (60 mM), 5-HT (1 μM) and histamine (10 μM) elicited maximal contractile effects of 2041.8 ± 94.3 (n = 66), 1798.0 ± 103.9 (n = 53) and 1382.6 ± 89.4 mg (n = 44) respectively. As already exposed in a previous work [46], the contraction induced by histamine is lower than that produced by KCl or 5-HT ($P < 0.05$; one-way ANOVA with Tukey post hoc test). After contraction by these agents, the relaxant effect of blockers of different Ca^{2+} channels was analysed (**Figure 1**). The relaxations induced by nifedipine on KCl and histamine contracted HUA were similar, however the effect on 5-HT contracted arteries was lower ($P < 0.05$). In general, all the relaxing drugs tested in this work have lower effects on HUA contracted by 5-HT than on contractions induced by KCl or histamine. On the other hand, relaxations induced by mibefradil (TTCC blocker) and APB (TRP channels blocker) were very small, independently of the contractile agent.

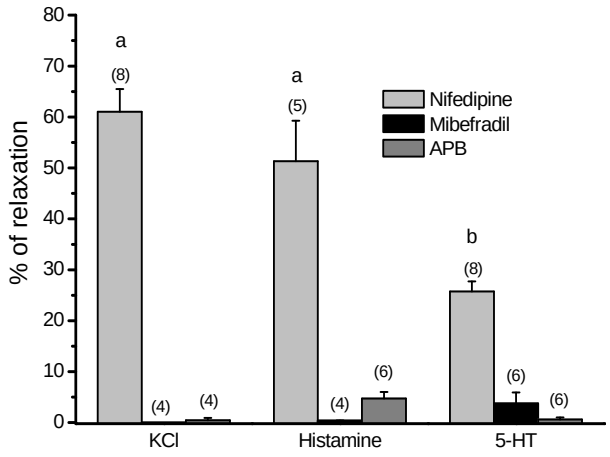

Figure 1. Effects of different Ca^{2+} channel inhibitors on HUA. Relaxation induced by nifedipine (10 μM), mibefradil (10 μM) and APB (100 μM) on contractions elicited by KCl (60 mM), histamine (10 μM) and 5-HT (1 μM). The bars represent the means and the lines the S.E.M. of the number of experiments indicated near the bars. Bars with different letters indicate significant differences in the effect of nifedipine ($P < 0.05$, one-way ANOVA with Tukey post hoc test).

Thus, TTCC or TRP inhibitors did not relax HUA and the relaxation induced by the inhibition of LTCC has been bigger when arteries were contracted by depolarisation or by histamine than by 5-HT receptor activation.

3.2. Effect of Cyclic Nucleotides in Ctracted HUA

The effect of cAMP increase on HUA contracted arteries has been analysed by using forskolin (adenylate cyclase stimulator) and rolipram (PDE4 inhibitor). The conjoint application of rolipram (1 μM) and forskolin (10 μM) have relaxed the contractions induced by KCl on 41.0% (**Figure 2**). In contrast, the effect of these two drugs applied together on 5-HT contracted arteries was only 10% (**Figure 2**). These drugs almost fully relaxed the HUA contracted by histamine (10 μM). Thus, the increase of cAMP have induced almost a full relaxation on histamine contracted HUA, 41% when arteries are contracted by depolarisation and a very small effect on 5-HT contracted arteries.

The effect of PKA inhibition on these relaxations has been analysed by using KTa (1 μM). The PKA inhibition induced a significant reduction on the forskolin plus rolipram effect in KCl and histamine contracted arteries ($P < 0.05$, **Figure 2)**, but was not efficient reducing this effect in 5-HT contracted arteries ($P > 0.05$; **Figure 2**). Besides, even in KCl and histamine contractions, the PKA inhibition did reduce in a tiny way the relaxation induced by the cAMP increase, suggesting that cAMP relaxation is partially independent of PKA and indicating the existence of another pathway linked to cAMP.

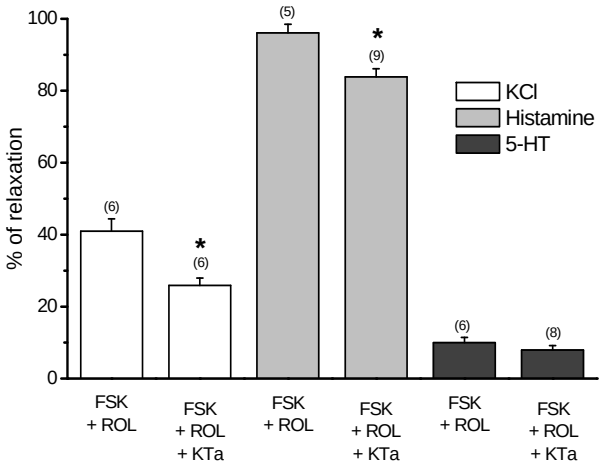

Figure 2. Relaxant effect of cAMP increase on HUA contracted arteries. Relaxation of HUA induced by the combination of forskolin (FSK; 10 μM) and rolipram (ROL; 1 μM) on contractions elicited by KCl (60 mM), histamine (10 μM) and 5-HT (1 μM). The effect of KTa (1 μM) on this relaxation is also shown. The bars represent the means and the lines the S.E.M. of the numbers of experiments indicated near the bars. Significant differences versus FSK+ROL effect are shown (*$P < 0.05$, Student-t test).

The effect of cGMP on HUA contracted arteries has been also analysed by using SNP (guanylate cyclase stimulator) and dipyridamol (PDE5 inhibitor). The bigger relaxant effect of conjoint application of dipyridamol and SNP has been obtained in HUA contracted by histamine (90.8%), being the effect on 5-HT- and KCl- contracted HUA considerably lower, 33.0% and 30.8% respectively (**Figure 3**). Thus, the increase of cGMP has induced almost a full relaxation (90.8%) on histamine contracted HUA, and around 30% of relaxation on arteries contracted by KCl or 5-HT.

The effect of PKG inhibition on these relaxations has been analysed by using KTg (1 μM). The PKG inhibition have induced a significant reduction on the SNP plus dipyridamol effect on histamine and 5-HT contracted arteries ($P < 0.05$; **Figure 3**), but the reduction induced in KCl contracted arteries was not significant ($P > 0.05$; **Figure 3**). As for PKA, the PKG inhibition did not completely reduce the relaxation induced by the cGMP increase, also suggesting the contribution of another pathway distinct than the PKG activation.

3.3. Effect of Phosphatase 2A Inhibition on Contracted HUA

HUA rings were contracted by KCl (60 mM), histamine and 5-HT, and the effect of OA (phosphatase 2A inhibitor; 5 nM) on these contractions was analysed.

Okadaic acid (5 nM) have relaxed the contracted HUA and this effect was bigger on contractions induced by KCl than when contraction were induced by histamine or 5-HT ($P < 0.05$; **Figure 4(a)**). Thus OA relaxes better the contractions induced by depolarisation, when LTCC are activated.

In KCl contracted arteries, the OA relaxation was similar than the induced by nifedipine. When these two drugs were applied together, the relaxant effect was not bigger than the relaxation induced by nifedipine or OA applied alone ($P > 0.05$; **Figure 4(b)**). However, the presence of forskolin and rolipram, drugs which increase the cAMP levels, significantly enlarged the OA effect ($P<0.05$; **Figure 4(b)**). Also, the presence of SNP and dipyridamol, drugs increasing the cGMP levels, significantly amplified the OA effect ($P < 0.05$; **Figure 4(b)**). Thus, the relaxant effect of LTCC inhibition and phosphatase 2A inhibition are not additive or synergic. However, the cyclic nucleotides have augmented the relaxation induced by PP2A inhibition.

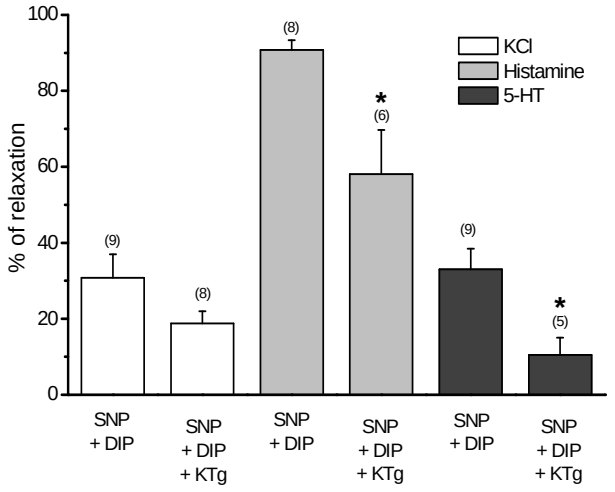

Figure 3. Relaxant effect of cGMP increase on HUA contracted arteries. Relaxation of HUA induced by the combination of SNP (10 μM; guanylate cyclase activator) and dipyridamol (DIP; 3 μM) on contractions elicited by KCl (60 mM), histamine (10 μM) and 5-HT (1 μM). The effect of KTg (1 μM) on this relaxation is also shown. The bars represent the means and the lines the S.E.M. of the numbers of experiments indicated near the bars. Significant differences versus SNP+DIP effect are shown (*P < 0.05, Student-t test).

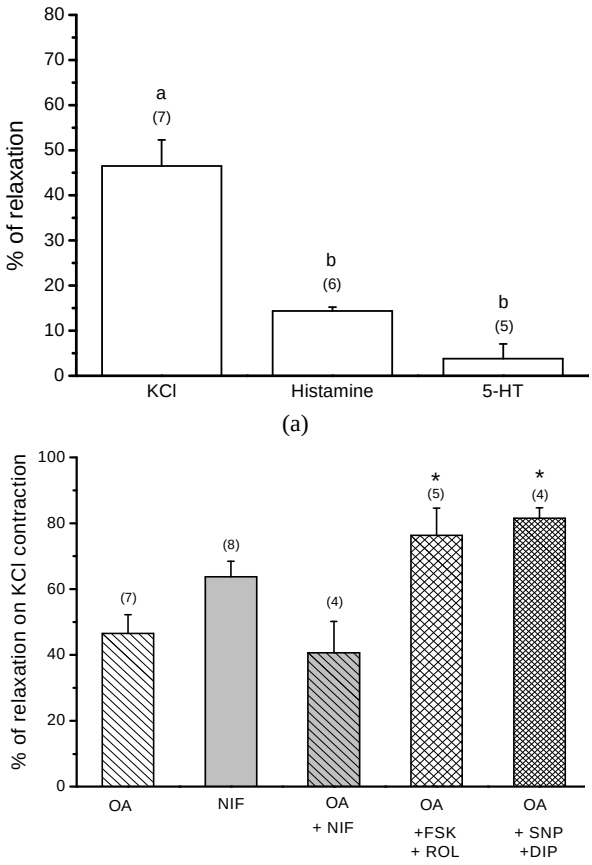

Figure 4. Relaxant effect of PP2A inhibition in HUA contracted arteries. a) Relaxation induced by OA (5 nM) on HUA contractions elicited by KCl (60 mM), histamine (10 μM) and 5-HT (1 μM); b) Effect of nifedipine (NIF; 10 μM), forskolin (FSK; 10 μM) plus rolipram (ROL; 1 μM) and SNP (10 μM) plus dipyridamol (DIP; 3 μM) in the OA relaxation. Statistical significant differences versus the OA effect are indicated (*P < 0.05, one-way ANOVA with Dunnet´s post hoc test).

3.4. Effect of Rock Inhibition in HUA Contractility

The role of different kinases in HUA contractility has been analysed by using the following inhibitors: Y27 (ROCK inhibitor; 10 μM); PD (ERK1/2 inhibitor; 50 μM); and SB (p38MAPK inhibitor; 25 μM).

Firstly, the effect of these inhibitors on HUA contractility was analysed in Ca^{2+} containing medium. In these conditions, ROCK inhibition did not induce significant relaxation ($P > 0.05$) of HUA contracted by KCl (0.3± 0.2%, n=8), by histamine (0.1 ± 0.1%; n=5) or by 5-HT (0.8±0.8%; n=6). Neither, in the same conditions, ERK1/2 inhibition did not induce significant relaxation ($P>0.05$) of HUA contracted by KCl (3.0 ± 1.5%; n=9), by histamine (1.7 ± 1.7%; n=5) or by 5-HT (1.1±1.1%; n=4). Also, in presence of extracellular Ca^{2+}, p38MAPK inhibition did not induce significant relaxation ($P > 0.05$) of HUA contracted by KCl (1.2 ± 1.0%; n=8), by histamine (1.1 ± 0.7%; n = 7) or by 5-HT (1.6 ± 1.0%; n=5). Thus, ROCK, ERK1/2 or p38MAPK inhibition did not affect the contraction induced either by KCl, histamine or 5-HT in presence of extracellular Ca^{2+}.

The effect of these inhibitors has been also analysed in absence of extracellular Ca^{2+} (0Ca medium). In these conditions KCl has induced sustained contractions (1013.6 ± 79.2 mg; n = 18) that were significantly lower than the induced in presence of Ca^{2+} (1859.8 ± 70.9 mg; n = 95)($P < 0.05$; Student-t test). The ERK1/2 or p38 MAPK inhibition did not significantly influence the contractions induced by KCl in absence of Ca^{2+} (**Figure 6(a)**). Besides, the inhibition of PP2A or LTCC did not affect the contractions induced by KCl in absence of extracellular Ca^{2+} (**Figure 6(a)**). However, ROCK inhibition by Y27 relaxed on 81.6% the contractions induced by KCl in Ca^{2+} free medium (**Figure 6(a)**). **Figure 5(a)** shows a record of an experiment in which KCl (60 mM) induces contraction of HUA in Ca^{2+}-free medium and Y27 relaxes this contraction.

On the other hand, in Ca^{2+} free medium histamine and 5-HT have elicited two step contractions characterized by a rapid phasic component, 2-3 min after the receptor stimulation, followed by a decrease of the contraction until a tonic component, 15-20 min after. The ERK1/2 or p38MAPK inhibition did not affect the phasic or tonic components of the contraction elicited by histamine or 5-HT ($P > 0.05$; **Figures 6(b)** and **6(c)**). However, ROCK inhibition significantly reduced the phasic and tonic components of the contraction elicited either by histamine or 5-HT ($P < 0.05$; **Figures 6(b)** and **6(c)**). **Figure 5(b)** shows a record of an experiment in which, in Ca^{2+} free medium, 5-HT elicited two step contractions characterized by a phasic and tonic components which were reduced after ROCK inhibition.

Thus, ROCK inhibition decreases the tension induced either by KCl, histamine or 5-HT in Ca^{2+} free medium.

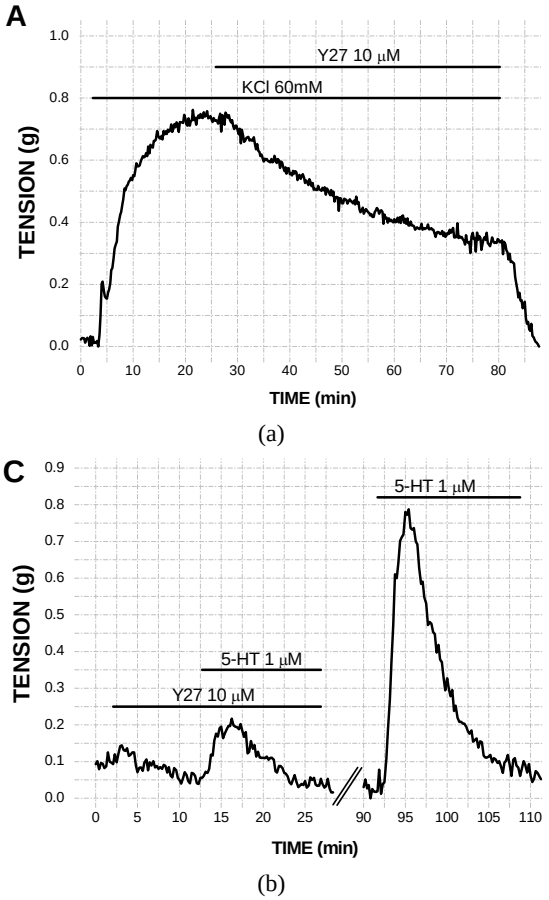

Figure 5. Original records of two tension experiments with HUA rings in Ca^{2+} free medium. a) Ca^{2+}-free medium contration of HUA induced by KCl (60 mM) which is relaxed by Y27 (10 μM); b) Ca^{2+}-free medium contraction of HUA induced by 5-HT (1 μM) in presence and in absence of Y27 (10 μM).

4. DISCUSSION

The present study investigated the involvement of some mechanism, such as different types of Ca^{2+} channels, cyclic nucleotides and different kinases, in the regulation of the HUA contractility. The elucidation of the precise mechanism regulating the contractility of this artery could be very important to detect potential therapeutic targets to treat HUA disorders such as preeclampsia.

We firstly investigated the relevancy of different types of Ca^{2+} channels on the regulation of HUA tonus. Our results show that the inhibition of LTCC relaxes contracted HUA, even if this effect does not reach 100% of relaxation. Other authors have previously reported that, among different Ca^{2+} channel blockers, nifedipine is the most potent umbilical vasodilator [18]. At the vascular level, it has been shown that nifedipine does not fully relax contractions induced by KCl. The existence of intracellular Ca^{2+} release when the arteries are contracted

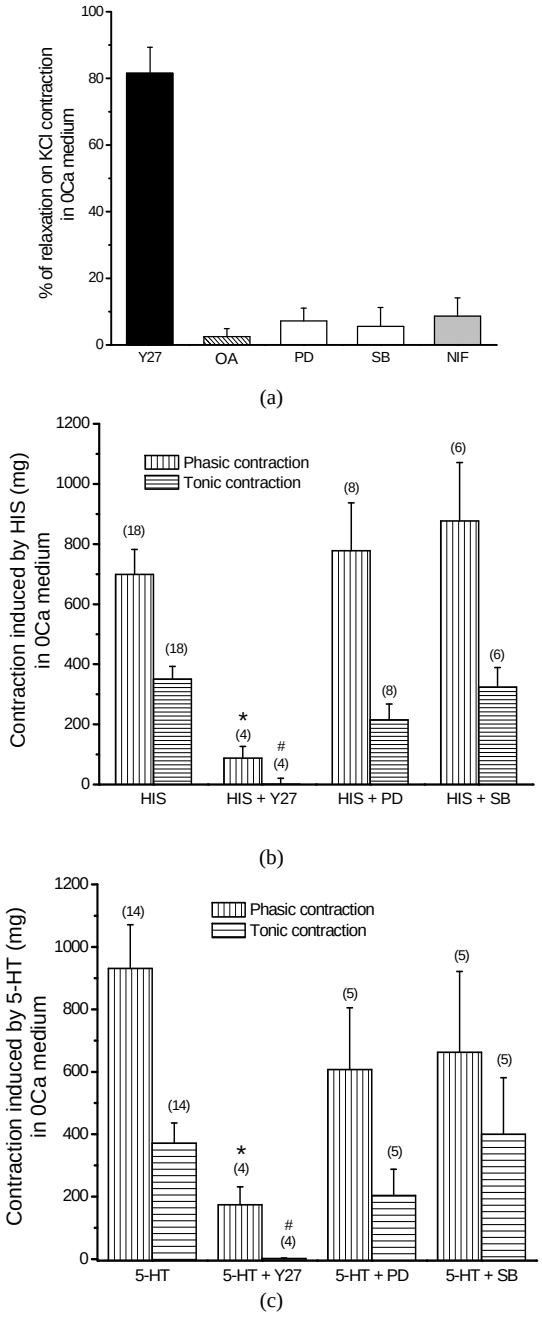

Figure 6. Relaxant effect of different inhibitors of kinases on HUA contracted in Ca^{2+}-free medium. a) Effect of Y27 (10 μM), OA (5 nM), PD (50 μM), SB (25 μM) and nifedipine (NIF; 10 μM) on KCl induced contraction in Ca^{2+}-free medium; b) Effect of Y27 (10 μM), PD (50 μM) and SB (25 μM) on the phasic and tonic contraction induced by histamine (10 μM) in Ca^{2+}-free medium; c) Effect of Y27 (10 μM), PD (50 μM) and SB (25 μM) on the phasic and tonic contraction induced by 5-HT (1 μM) in Ca^{2+}-free medium. The bars represent the means and the lines the S.E.M. of the numbers of experiments indicated near the bars. Statistical significant differences versus the effect in absence of inhibitor are indicated (*P < 0.05, one-way ANOVA with Dunnet´s post hoc test).

by KCl has been suggested as responsible of the lack of a full relaxant effect of LTCC inhibitors [7]. In this sense, and as we will discuss later, the ability of KCl to induce HUA contractions in Ca^{2+}-free medium could be due to stimulation of intracellular Ca^{2+} release. The relaxant effect of nifedipine was stronger when HUA were contracted by depolarization or by histamine than in arteries contracted by 5-HT. Mikkelsen *et al*. also have observed that, in human pulmonary arteries, the contraction induced by 5-HT is more resistant to nifedipine than the contraction mediated by depolarization [8]. On the other hand, several studies demonstrated that TTCC and TRP channels are involved in VSM contraction in different arteries [5,9,10,47]. However, our results show the absence of effect of mibefradil and APB, suggesting that Ca^{2+} entry through TTCC and TRP channels does not contribute to HUA contraction.

The precise regulation of the intracellular levels of cAMP and cGMP plays an important role in many physiological processes, including vascular smooth muscle contractility [32]. However, the mechanisms by which increases in cAMP and cGMP concentration lead to artery relaxation are still unclear. Some authors have reported that forskolin, a direct stimulator of adenylate cyclase, induced relaxation of different human vessels such as dorsal artery [34], pulmonary artery [35], placental vessels [36] and umbilical artery [37]. On the other hand, among the four families of PDE expressed in this smooth muscle (PDE1, PDE3, PDE4 and PDE5), PDE4 has been shown as the key enzyme involved in the regulation of HUA relaxation associated to cAMP [37]. In this sense, we used forskolin (adenylate cyclase stimulator) and rolipram (PDE4 inhibitor) as drugs that can elicit the maximal increase in cAMP levels in HUA smooth muscle cells. The conjoint application of both drugs elicited different degree of relaxation depending on the contractile agent used. When applied together, these drugs almost fully relaxed histamine contracted HUA, but only 41% and 10% of relaxation was obtained on HUA contracted by KCl and 5-HT, respectively. We have previously shown that 5-HT_{2A}, 5-$HT_{1B/1D}$ and 5-HT_7 receptors are present in HUA smooth muscle cells. The HUA contraction induced by 5-HT are mainly mediated by the activation of 5-HT_{2A}, which activation increases IP_3 levels, and 5-$HT_{1B/1D}$ receptors, which activation inhibits adenylate cyclase [48]. Concerning the histamine receptors, H_1 receptor activation induces contraction and H_2 and H_3 receptors activation mediates HUA relaxation through the increase of cAMP intracellular levels [48]. According with these previous findings, the activation of different 5-HT receptors in HUA inhibits adenylate cyclase and the activation of different histamine receptors induces contraction, but also induces a small increase of cAMP levels due to H_2 and H_3. Thus, histamine contractions are more susceptible to activators

of adenylate cyclase or to PDE4 inhibitors than 5-HT contractions. On the other hand, apparently KCl does not affect adenylate cyclase activity and the effect of forskolin and rolipram has been lower than in histamine-contracted arteries and bigger than HUA contracted by 5-HT.

Is well known that NO activates soluble guanylate cyclase increasing cGMP levels, which induces vasodilatation [38], including HUA vasodilatation [37,39]. On the other hand, PDE5 has been shown as being the key enzyme involved in the regulation of HUA relaxation associated to cGMP [37]. In this sense, we used SNP (guanylate cyclase stimulator) and rolipram (PDE5 inhibitor) as drugs that can elicit the maximal increase in cGMP levels in HUA smooth muscle cells. As for cAMP, the conjoint application of these drugs has elicited different degree of relaxation depending on the contractile agent used. The biggest relaxant effect was observed in contraction induced by histamine, followed by contractions produced by 5-HT and by KCl depolarization. Numerous authors have described that cGMP induced vasodilatation is mediated by activation of potassium channels in different arteries [40,41], including HUA [39]. The regulation of voltage-dependent potassium channels (K_V) by KCl and 5-HT could be responsible of these differences. It has been described that vascular contraction induced by KCl is mainly due to the influx of extracellular Ca^{2+} via voltage-dependent Ca^{2+} channels [1], but Kv channel inactivation at high potassium concentrations (60 mM) was also demonstrated in some blood vessels [49]. Recently, using patch clamp techniques, some authors have demonstrated that 5-HT decreases Kv channel activity in cells of rat pulmonary [50] and mesenteric [51] arteries. Thus, as the cGMP relaxant effect seems to be mediated by potassium channels activation, this effect is lower when HUA are contracted by KCl and 5-HT because these agents inhibit these channels.

Our results have shown that the PKA inhibition induced a significant reduction on the forskolin plus rolipram effect in KCl and histamine contracted arteries, even if this effect is small. The relaxant effect of cAMP increase in 5-HT contracted arteries was very low (around 10%) and KTa have failed to significantly decrease the relaxations induced by forskolin plus rolipram. Also, the PKG inhibition induced a significant reduction on the SNP plus dipyridamol effect in histamine and 5-HT contracted arteries. The analysis of these results suggests that both PKA and PKG are involved in HUA relaxation mediated by cAMP and cGMP respectively. However, the contribution of these kinases is very small and in some cases was not significant. These results suggest that the relaxation induced by cyclic nucleotides is not totally mediated by the activation of their respective kinases and other mechanisms can be involved as described by other authors [44,45]

As we mentioned before, LTCC are critically important for regulating HUA contraction. It has been described that LTCC can be dephosphoralyted by PP2A, and inhibition of PP2A was found to result in changes in functional properties of the LTCC [21]. However, there is small number of studies on this matter in smooth muscle and the direction of this modulation has remained somewhat controversial. Some authors have shown that in tracheal smooth muscle PP2A does not modulate LTCC [19]. Also, it has been shown a dual effect of phosphatase inhibitors on Ca^{2+} channels from intestinal smooth muscle cells [20]. At the vascular level, Groschner *et al*. have demonstrated that, in human umbilical vein, when PP2A is inhibited by OA there is increase of LTCC activity [21]. Our results show that PP2A inhibition produces relaxation of HUA, namely when this artery is contracted by KCl and histamine. This relaxant effect is bigger in arteries contracted by KCl. Our results also show that in KCl contracted arteries, the OA effect is similar to the nifedipine effect. When applied together, the relaxant effect is not bigger than the relaxation induced by nifedipine or OA applied alone. These results suggest that PP2A could activate LTCC in HUA. On the other hand, the increase of cAMP or cGMP induced by the conjoint application of cyclase activators and PDE inhibitors significantly increased the OA effect. These results suggest that the relaxation induced by PP2A inhibition is independent of the cyclic nucleotide pathway.

We also have analyzed the effect of some kinases on HUA contractility. Our results show that ROCK, ERK1/2 or p38MAPK inhibition does not affect the contraction induced by KCl, histamine or 5-HT in Ca^{2+}-containing extracellular solution. Other authors have obtained significant relaxation of HUA contracted by 5-HT after ROCK inhibition, but using higher concentrations of Y27 (100 μM) which can inhibit also myosin light chain kinase directly [22]. Also, Tasaki *et al*. observed that SB significantly inhibits 5-HT-induced contractions in rat aorta [30]. Some authors indicated that 5-HT induces contractions of rat aortic smooth muscle by activating the MAPK pathway [29]. Also, activation of p38 MAPK [30] and Erk MAPK [31] by 5-HT have been shown in rat aorta.

Our results suggest that in the contractions induced by KCl, histamine and 5-HT in presence of Ca^{2+} there is not involvement of pathways concerning ROCK, ERK1/2 or p38MAPK. Sakurada *et al*. have shown the existence of a Ca^{2+}-dependent Rho stimulation mechanism in VSM from rabbit aorta which is activated by excitatory receptor agonists [23]. However, in HUA our results exclude this possibility, because in presence of extracellular Ca^{2+} ROCK does not seem to be activated by KCl, histamine or 5-HT.

On the other hand, in absence of extracellular Ca^{2+}, histamine and 5-HT elicited contractions of HUA characterized by two components, a rapid phasic contractile

component, 2-3 min after stimulation by the agonist, followed by a decrease of the contraction until a tonic component, 15-20 min after. The initial transient component has been associated with Ca^{2+} release from the sarcoplasmic reticulum, whereas the tonic component seems to be dependent on the increase on extracellular Ca^{2+}. Both contractile responses are dependent on the Ca^{2+} sensitization and two main pathways have been implicated in this phenomenon: the inhibition of myosin light chain phosphatase (MLCP) by ROCK; and the phosphorylation of the thin filament proteins by p38MAPK and ERK1/2 [5,52]. In contrasts, KCl elicited sustained contractions of HUA in absence of extracellular Ca^{2+}. This effect could be induced by a progressive Ca^{2+} release from intracellular Ca^{2+} stores and/or an increase of Ca^{2+} sensitization. These data agree with the obtained by Tufan *et al*. in HUA, which have suggested that Ca^{2+} independent isozymes of protein kinase C may also be involved in the contractions produced by KCl in absence of extracellular Ca^{2+} [1]. Some authors have also suggested that depolarization by KCl induces intracellular Ca^{2+} release in human arteries [7]. Besides, it has been described that KCl depolarization increases Ca^{2+} sensitization by ROCK activation in smooth muscle cells from rabbit arteries [24]. As expected, in absence of extracellular Ca^{2+}, nifedipine did not relax the contractions elicited by KCl. Also in these conditions, OA did not relax the contractions elicited by KCl. Once more, these data suggest a functional relationship between the PP2A and LTCC in the regulation of HUA contractility.

Concerning the kinases, the ERK1/2 and p38MAPK inhibitors did not reduced or relax the contractions induced by KCl, histamine or 5-HT in absence of extracellular Ca^{2+}. Thus, these results demonstrate that KCl-induced contractions are not linked to the activation of ERK1/2 and p38MAPK. However, in Ca^{2+}-free medium, the ROCK inhibition significantly relaxed the contractions induced by KCl and reduced the phasic and tonic components of the contraction elicited either by histamine or 5-HT. Similar results were obtained by Ark *et al*. when HUA were contracted by 5-HT in Ca^{2+} free medium [22]. Our data demonstrate that ROCK is capable of mediating the contractile response after stimulation of a receptor agonist or by depolarization. Thus, in absence of Ca^{2+}, the sustained contractions elicited by KCl and the phasic and tonic components of the contraction induced by histamine or 5-HT depend on ROCK activation. Consequently, these contractions depend of Ca^{2+} sensitization by the inhibition of MLCP by ROCK. Our data show that the relevancy of the Ca^{2+} dependent or independent mechanism in HUA varies in function of the extracellular Ca^{2+} level, and further experiments are necessary to deeply study this dependence. On the other hand, our results suggest that HUA is a good sample to study the Ca^{2+} sensitization mechanism induced by ROCK.

In conclusion, our results demonstrate that the LTCC are the main way for Ca^{2+} entry and is involved in HUA contractions induced by depolarization or by agonists. A positive regulation of LTCC by PP2A seems to occur in HUA smooth muscle cells. Cyclic nucleotides are involved in HUA vasodilatation. The relaxant effect of cyclic nucleotides is partially due to the activation of their respective kinases, but other pathways are also involved. HUA is able to contract independently of Ca^{2+} influx by activating the ROCK pathway and/or due to intracellular Ca^{2+} release.

5. ACKNOWLEDGEMENTS

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